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ABSTRACT

Plant tissue culture has developed into a valid botanical discipline and is considered a key area of biotechnology, but it has not been a key component of the science curriculum because of the expensive and technical nature of research in this area. This manual presents a number of activities that are relatively easy to prepare and perform. The activities also provide a problem-solving approach to learning as a means of conveying basic content and science process skills. Units include: (1) The Process of Science; (2) Aseptic Technique; (3) Culture Methods; (4) Regeneration; (5) Preparation of Tissue Culture Media; and (6) Cell Culture Applications. Appendices include instructor's notes, a supplies directory, case study discussions, statistical tables, and student investigations. (WRM)

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PLANT TISSUE CULTURE STUDIES

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PLANT TISSUE CULTURE STUDIES

- Experimental Design
- Aseptic Technique
- Basic Culture Methods
- Regeneration
- Media Preparation
- Cell Culture Applications

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Printed in the United States of America by Automated Graphic Systems, White Plains, Maryland.

On the cover ... The cover photo is an illustration of oat (Avena sativa) *leaf protoplasts, as seen under high power (430X), stained with Evan's Blue reagent. Intact protoplasts exclude the dye (see Exercise 25).*

The cover photo and illustrations throughout the book are courtesy of George W. Fahy, Michael Fahy, and Robert A. Smith. NABT wishes to thank them for their contributions.

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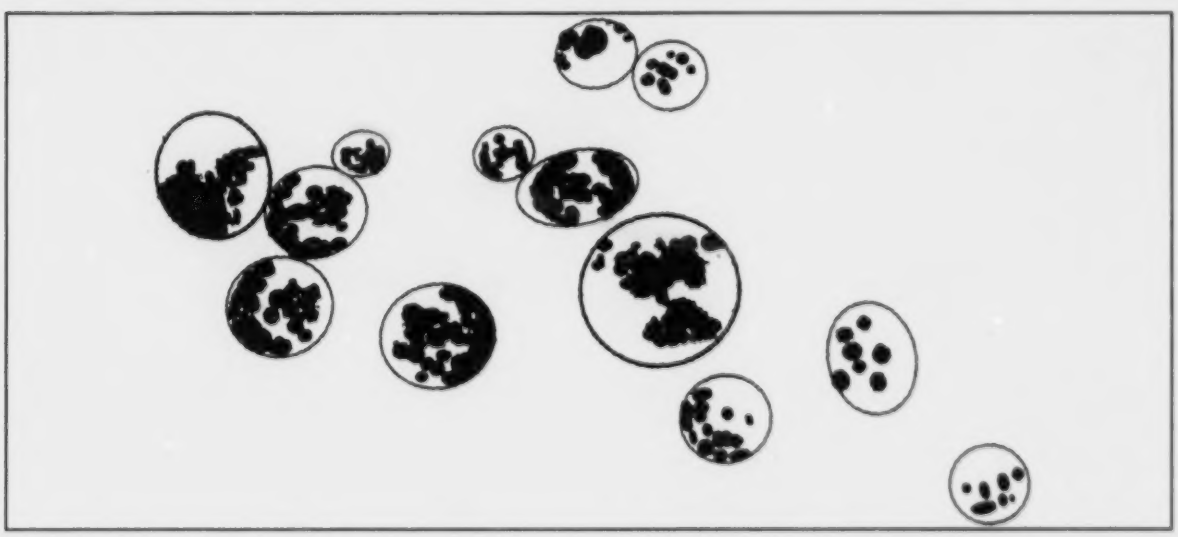
PREFACE

Plant tissue culture is the growing of isolated plant parts, aseptically, on appropriate media (Steward 1983). It includes basic propagation techniques, such as *organogenesis, embryogenesis,* and *shoot tip culturing.* It also includes specialized techniques, such as *protoplast fusion* and *genetic transformation,* used for production of new plant varieties or for insertion of foreign genes into fast-growing plants to be used as efficient manufacturers of specific chemicals. This is why plant tissue culture has developed into a valid botanical discipline and is considered a key area of biotechnology for the next century. It is appropriate subject material for basic biology courses; however, it generally is not represented in biology courses or curricula. The requirement of aseptic conditions, usually meaning use of an expensive laminar flow unit, has been a major obstacle. Other obstacles include difficulty in obtaining specific plant material used for some experiments, difficulty in media preparation, and long incubation times before obtaining results.

One purpose of this manual is to attempt to remedy these obstacles. A number of activities are presented that are relatively easy to prepare and perform. If the instructor provides appropriate modeling, these experiments can be done on open bench tops with a high degree of success. In addition, the plant materials used in these experiments are easily obtained from biological supply houses or local stores. They grow rapidly in culture, allowing reading of results in six weeks or less. Finally, for purpose of comparison, only two media are used in this manual: Gamborg's B-5 medium and Murashige and Skoog Salt Base. These are available commercially, easing the task of preparation, yet there is ample opportunity for experimental modification.

Another purpose of this manual is to provide students with a problem-solving learning approach as a means of conveying basic content and science process skills. The problem-solving learning approach used is that of the investigative laboratory. There appears to be support in the literature for the idea that a direct interaction with science process skills, which includes the ability to raise questions, the ability to generate multiple tentative answers, and the ability to test these answers, is desirable (Peterson & Jungck, 1988) and even essential (Lawson, 1992) for developing these skills.



Some background for this process is provided in introductory biology texts. In addition, many investigative laboratory manuals allow students to apply many aspects of this process; however, most texts cover the scientific method without including much in the way of statistical analyses. Yet many modern biological problems rely upon quantitative observations. While it is still necessary to examine the data for patterns prior to statistical analysis, the function of statistical tests is to provide an objective means for analyzing and interpreting data and then drawing conclusions based upon the data. This is the way many biologists will be using the science process throughout their lives — with the statistical analyses. Thus, the emphasis in teaching it this way is appropriate.

Unit 1 presents a number of exercises intended to provide this background. In Exercise 1, students gain practice *posing questions*. Exercises 2 through 5 are case study activities that provide samples of *specific experimental designs*. These designs allow analysis by several commonly used statistical tests. Once students gain practice using these specific designs and statistical tests, they will be better able to apply these solutions to problems of their own. In Exercise 2, a *Student t test* is used to compare measurements of a variable from two samples. In Exercise 3, a *single factor analysis of variance* is used to compare measurements of a variable from three or more samples. In Exercise 4, a *two-factor analysis of variance* is used for simultaneous analysis of more than one factor. In Exercise 5, a multiple comparison procedure known as the *Tukey test* is presented. This test may be used for further analysis of one- and two-way analyses of variance. Finally, Exercise 6 provides some guidelines for *poster presentations*. A sample poster is illustrated. Presentations provide opportunities for modifications of ideas (Peterson & Jungck, 1988).

In succeeding units, activities are presented to provide background and theory in additional plant tissue culture areas, to provide opportunities for students to apply this process of science, and hopefully, to serve as springboards for student-generated projects. It is recommended that students apply each experimental design at least once. Subsequently, the design of each exercise allows the instructor the flexibility to present them with or without statistical analysis.

This manual also may be used as a sourcebook of basic plant tissue culture techniques, experimental designs and statistical tests, and poster presentation guidelines in other plant courses or for science fair projects or undergraduate research. Many students who have taken the plant tissue culture course for which this manual was designed have continued their studies using these basic techniques and presented their findings in other classes, at scientific meetings, and in peer-reviewed journals.

References

Lawson, A.E. (1992). The development of reasoning among college biology students — A review of research. *Journal of College Science Teaching, 21,* 338-344.

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SAFETY CONSIDERATIONS

The following guidelines should be followed for your protection in the laboratory. In addition, the school Safety Officer or other personnel familiar with the *Workplace Hazardous Materials Information System* should be consulted for instructions regarding biohazardous and other waste and for specific instructions regarding handling of hazardous chemicals and organisms.

✔ Protective clothing, including a lab coat and shoes, must be worn before starting an experiment. Safety eyewear should be worn while performing laboratory exercises for protection from harmful chemicals.

✔ Confine long hair and other loose items that could catch fire or cause spills or contamination.

✔ No materials should be placed in the mouth during lab. There will be no eating, drinking, smoking, or mouth pipetting. Use a mechanical pipetting device. The instructor will demonstrate the proper attachment of the pipet to the device.

✔ Notify the instructor of broken glassware. The instructor will dispose of it in the proper broken glassware container.

✔ Notify the instructor of any accidents, reagent spills, or other potential safety problems.

✔ Dispose of all contaminated materials, including used cultures, pipets, gloves, and any materials contaminated with blood or other body fluids, in the proper hazardous materials container.

✔ Do not use flammable liquids near flames.

✔ Use autoclave gloves when working with hot objects.

✔ The Material Safety Data Sheets for all reagents provided by the manufacturer should be maintained in a folder in the lab. These sheets include, among other important information, potential health effects and first aid measures for each reagent. Consult your school's Safety Officer.

✔ All reagents must be stored and disposed of properly. Consult your school's Safety Officer.

✔ A fume hood must be used when working with many reagents, such as when spraying thin layer chromatography plates. Consult your school's Safety Officer.

✔ Do not remove cultures, reagents, equipment, or other materials from the lab.

✔ Do not perform experiments without supervision. The instructor must explain safety procedures associated with the activity.

✔ Store items, such as coats and books, in designated areas and not at your work station.

✔ Wear protective gloves when handling materials contaminated with bacteria, fungi, blood, or other body fluids.

✔ Wash your hands with disinfectant soap before leaving the laboratory area.

✔ Note fire exits and the placement of fire blankets, eyewashes, chemical showers, and other safety devices.

UNIT I – THE PROCESS OF SCIENCE

One aspect of science is that it is a method of problem solving. The reasoning skills used to solve scientific problems are considered generally useful and are not just tools for scientists. This problem-solving aspect of science actually involves a number of steps that are sometimes referred to as the **process of science**.

Problem Posing, Problem Solving

The science process skills include **asking questions, generating multiple tentative answers, and testing these answers** (National Science Foundation, 1989). Hands-on courses, in which students are given the opportunity to design and conduct their own investigations, appear to be successful at improving these reasoning skills (Lawson, 1992).

Persuasion

In addition to problem posing (asking questions) and problem solving (multiple tentative answers, testing these answers) skills, scientists must participate in the process of **persuasion**. Science is not finished until the results are reported, and the scientist has convinced his/her peers as to the reasonableness of the work (Peterson & Jungck, 1988).

The Science Process

The validity of new experimental findings is to be measured in terms of other experiments (Conant, 1951). Scientific articles are read to learn what is already known about a problem. Reading may raise more questions (**problem posing**) than it answers. Possible answers (**hypotheses**) to these questions are suggested and tested (**problem solving**). Then findings are presented. The **presentation** increases understanding of the problem and allows others to test questions that the work raises. This brings the process full circle. All of this may lead to finding a solution to the problem.

In this unit, the science process will be introduced. Exercise 1 examines problem posing. Exercises 2 through 5 are case study investigations that demonstrate several specific problem solving techniques. Exercise 6 concentrates on persuasion.

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Conant, J.B. (1951). *Science and Common Sense.* New Haven, CT: Yale University Press.

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Peterson, N.S. & Jungck, J.R. (1988). Problem-posing, problem-solving and persuasion in biology education. *Academic Computing, 2,* 14-17 & 48-50.

1. Problem Posing

Objectives

1. To understand and be able to describe the relationship between what is already known about a topic and how **general** and **specific questions** are generated.

2. To gain practice developing **specific questions** for investigation.

Background

Before one can develop questions about a particular topic, one must first determine what is already known about it. This requires examining various texts on the topic as well as reviewing the primary literature (original scientific papers). This provides a framework upon which questions may be generated for investigation. One way to start this process of problem posing is to examine the individual (introduction, methods, results, and discussion) sections of several primary references. During this process, it will probably become apparent that each author approached a particular problem differently. For example, they may have used different procedures to disinfect plant material (see Exercises 2 and 8), or they may have sterilized their media differently (see Exercises 20 and 21), or they may have modified their media using different supplements (see Exercises 3, 13, and 23). These differences frequently are a source for problem posing. The authors may raise questions that need additional study in the discussion section of their articles.

In addition, after selecting a particular problem to study, one may find that he or she does not have access to the same equipment or plant material as in the original article. This further modifies the problem. Also, one may decide to use a different experimental design (see Exercises 2 through 4) to provide additional data. Each modification adds to the originality of the work. These modifications are not made merely for the sake of change; rather, it is hoped that these differences will add insight to the problem.

Procedure

1. Read the background information in Exercises 2 and 3.

2. Answer the questions for this exercise.

Report 1
Problem Posing

Name:

1. What is the **general problem** that is posed in Exercise 2?

2. What is the **specific problem** that is posed in Exercise 2?

3. Some workers prefer 70% ethanol or 70% isopropyl alcohol for disinfection of plant material. Based on this information, suggest several of your own **specific questions** for investigation.

a.

b.

c.

4. Compare the problem-solving approaches of Exercises 2 and 3. Based on this information, suggest a **specific question** for investigation utilizing the new experimental design.

2. Problem Solving Case Study: Student *t* Test

Objectives

1. To gain practice using a specific problem-solving approach known as the **Student *t* test**.

2. To recognize when it is appropriate to apply this test.

Background

Problem Posing

One major source of contamination in plant tissue culture is the plant material itself. Bacterial and fungal contaminants cover the surfaces of plants. The inside tissues usually do not contain these organisms. Viruses may still be present, but no steps are taken to remove them except for specialized procedures such as meristem culture. Thus, the disinfection process is aimed at removal of surface contamination.

For this purpose, ethanol and bleach alone or in combination have been used. For example, petioles from sweet potatoes were disinfected by exposure to 85% ethanol, followed by exposure to 1.5% sodium hypochlorite (Eilers et al., 1988). On the other hand, Kao and Michayluk (1980) used only 60% ethanol for disinfection of alfalfa leaves; however, Dos Santos et al. (1980) used only 5% bleach for disinfection of alfalfa leaves. Regardless of the treatment, the surfaces may not be sterile since alcohol is ineffective against spores (Larson & Morton, 1991), and bleach is only partially effective against spores (Dychdala, 1991).

Since bleach is partially effective against spores, it should be more effective than alcohol for disinfection of plant tissues. The **general problem**, then, is to compare the effectiveness of these two commonly used disinfectants. One approach to this problem is to use an experimental design that would allow analysis of data using a Student *t* test (Campbell, 1989; Dowdy & Wearden, 1991; Zar, 1996).

Problem Solving

Many scientific problems require that the specific problem-solving approach (the specific question, hypotheses, prediction of results, experimental design, collecting, analyzing and interpreting results, and then drawing conclusions) be designed to take advantage of a specific statistical procedure. The reason for this is that there is a natural variation, due to chance or random error, in any mass of raw data collected. When comparing two or more groups of data, and there appears to be a difference between the groups, the question then becomes, "Is this difference real or is it due to random error?" Statistical treatments allow us to determine this to a certain level of probability. One of the simplest problem-solving approaches is one that allows comparison of two independent samples, the Student *t* test.

The Specific Question

Since bleach is partially effective against spores, it should be more effective than alcohol for disinfection of plant tissues (Brehm et al., 1996). The general problem is to compare the effectiveness of these two disinfectants. To answer this, a **specific question** must first be posed. Following a review of a general reference on the topic of disinfection (Block, 1991), Brehm et al. (1996) decided to compare the efficacy of **95% ethanol** to **20% bleach** using a Student *t* test.

The Hypotheses

For statistical analysis, null and alternative hypotheses are stated. The **null hypothesis** for this experiment is "there is no difference in efficacy of 95% ethanol compared to 20% bleach as a disinfectant." Selection of an **alternative hypothesis** is more difficult. There are two types of alternative hypotheses: directional (one-tailed) and nondirectional (two-tailed). A **directional hypothesis** is justified if the differences between means of the bleach and alcohol populations are likely to be only in one direction (Schmidt, 1979). Since bleach, but not

alcohol, is effective against spores, a directional hypothesis is appropriate. Thus, the alternative hypothesis is "bleach is more effective than alcohol as a disinfectant."

If there was reason to suspect that ethanol would be just as likely to be the superior disinfectant, then a **nondirectional alternative hypothesis** would have been stated. That is, the alternative hypothesis would have been "there is a difference in efficacy of bleach and ethanol as disinfectants."

The Experimental Design

1. The medium used was Murashige and Skoog salt base (available from Carolina Biological Supply Company and other similar biological supply houses) containing 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 100 mg/L inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCL, 0.4 mg/L thiamine HCL, 2 mg/L glycine, 1 g/L casein hydrolysate, 30 g/L sucrose, and 8 g/L agar.

   The medium was sterilized by autoclaving, and 20 ml was dispensed into each 100 mm x15 mm petri plate.

2. Sweet potatoes *(Ipomoea batatas)* were scrubbed under running tap water to remove residual soil.

3. The skin was removed, and the potato was cut transversely into slices about 15-mm thick.

4. Half the slices were disinfected for 10 minutes using 20% (v/v) bleach and then rinsed with sterile distilled water.

5. The remainder slices were disinfected for 10 minutes using 95% (v/v) ethanol and then rinsed with sterile distilled water.

6. Using separate sterile instruments for each treatment, explants 10 mm in length and 5 mm in diameter were removed from the slices.

7. Five explants from each slice were placed onto each agar plate.

8. Explants were cultured for four weeks at 25° C in a growth chamber and then examined for contamination (Figure 2.1).

9. The experiment was repeated 15 times.

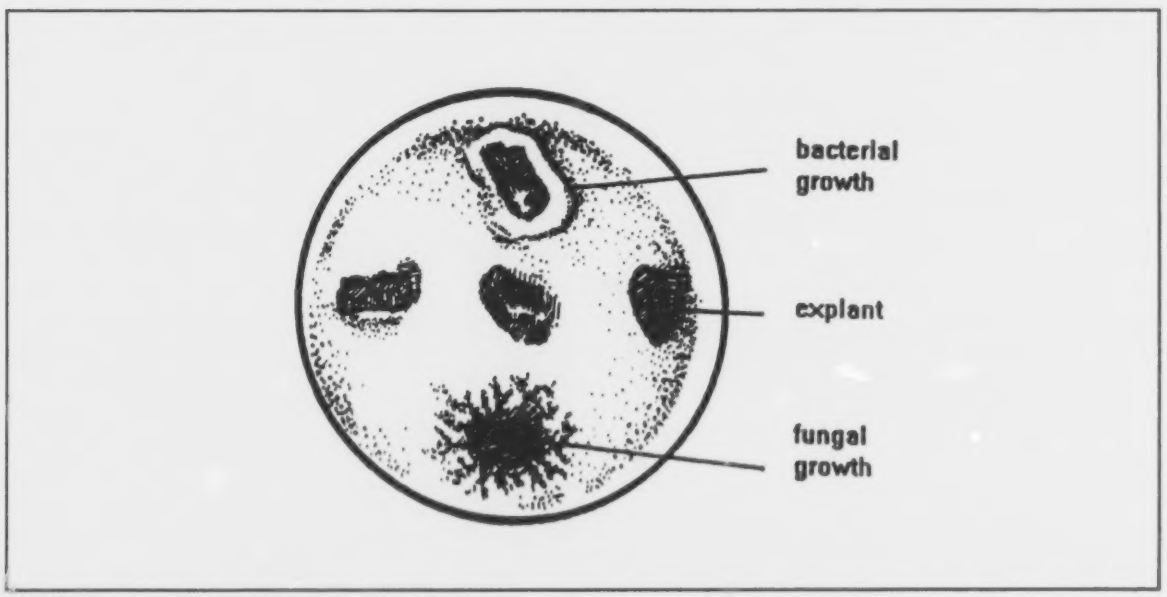

Figure 2.1. After four weeks of incubation, bacterial or fungal growth will be evident surrounding contaminated explants.

The Results

After four weeks of incubation, Brehm et al. (1996) collected the data presented in Table 2.1 on the opposite page.

References

Block, S.S. (1991). *Disinfection, Sterilization, and Preservation* (4th ed.). Philadelphia, PA: Lea & Febiger, 131-151.

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Zar, J.H. (1996). *Biostatistical Analysis* (3rd ed.). Upper Saddle River, NJ: Prentice-Hall, Inc.

Procedure

1. Examine the data in Table 2.1 and answer Question 1 on the result sheet.

2. Now, analyze the data using the formulas in Figure 2.2. Record all calculations on the result sheet.

   - First, use the *F* test to determine whether the variances of the two samples differ significantly. The *F* value should be calculated by dividing the larger by the smaller variance.

   - Compare the calculated value of *F* to the critical value of *F* for a one-tailed hypothesis, at the 0.05 level of significance (see Appendix D). To do this, determine the degrees of freedom for both the numerator and denominator (n-1). If this value does not appear in the table, the critical value with the next lower *df* is used. (The critical value of *F* at other levels of significance may be obtained from tables presented in texts of statistical analysis; e.g., Zar, 1996.)

   - If the calculated value of *F* is less than the critical value of *F*, accept the null hypothesis. That is, the variances are equal, and the Student *t* test must be used.

   - If the calculated value of *F* is equal to or greater than the critical value of *F*, reject the null hypothesis. That is, the variances are not equal, and the *t'* test must be used.

   - Now perform the appropriate *t* test. Compare the calculated *t* value to the critical value of *t* at the 0.05 level of significance (see Appendix D). Since the hypothesis is directional, a table for a one-sided test is used. (The critical value of *t* at other levels of significance may be obtained from tables pre-

Table 2.1. Results comparing the effectiveness of 95% ethanol and 20% bleach as disinfectants.

Trial	Number of Uncontaminated Explants	
	95% Ethanol	20% Bleach
1	0	5
2	1	5
3	4	4
4	2	5
5	4	5
6	2	4
7	5	5
8	5	5
9	4	4
10	5	5
11	5	5
12	5	5
13	4	5
14	5	5
15	5	5
Mean	3.73	4.8
Sample variance	2.78095	0.17143

sented in texts of statistical analysis; e.g., Zar, 1996.)

- If the calculated value of t is less than the critical value of t, accept the null hypothesis. That is, there is no difference in efficacy of the disinfectants.
- If the calculated value of t is equal to or greater than the critical value of t, reject the null hypothesis. That is, the number of uncontaminated explants obtained with bleach treatment is significantly more than the number obtained with ethanol treatment.

3. Compare the conclusion following statistical analysis with the conclusion made initially.

F test

$$F = \frac{s_1^2}{s_2^2} \quad \text{or} \quad F = \frac{s_2^2}{s_1^2} \qquad s^2 = \frac{(X - \bar{X})^2}{n\text{-}1}$$

is the variance for each group

Student *t* test (when σ_1^2 and σ_2^2 are unknown but assumed equal)

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{s_p^2/n_1 + s_p^2/n_2}}$$

$\bar{X}_1$ and $\bar{X}_2$ are the means for each group.

s_p^2 is the pooled variance [$(s_1^2 + s_2^2)/2$].

n_1 and n_2 are the sample sizes for each group.

$df = n_1 + n_2 - 2$

***t'* test** (when σ_1^2 and σ_2^2 are unknown but assumed unequal)

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{s_1^2/n_1 + s_2^2/n_2}}$$

$\bar{X}_1$ and $\bar{X}_2$ are the means for each group.

s_1^2 and s_2^2 are the variances for each group.

n_1 and n_2 are the sample sizes for each group.

$$v = \frac{\left(\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}\right)^2}{\frac{\left(\frac{s_1^2}{n_1}\right)^2}{n_1 - 1} + \frac{\left(\frac{s_2^2}{n_2}\right)^2}{n_2 - 1}}$$

degrees of freedom for *t'* test.

Figure 2.2. Formulas for *F* test, Student *t* test, and *t'* test (Zar, 1996).

Report 2
Problem Solving Case Study
Student *t* Test

Name:

---

1. Before performing the statistical analysis, which treatment appears to be most effective, if any?

2. Record the calculations for the *F* test here. Are the variances for the two samples equal?

3. Record the calculations of the appropriate *t* test here. What is your conclusion?

4. Compare your conclusion following statistical analysis with the one made initially.

5. What is the practical significance of your conclusion?

6. What is the influence of having different pairs of students collect data instead of one pair of investigators?

7. Would a larger number of replicates influence your outcome? Explain.

* *Refer to Appendix C to compare your analysis and conclusions with those of the original authors.*

---

3. Problem Solving Case Study: One-Way Analysis of Variance

Objectives

1. To gain practice using a specific problem-solving approach known as the **one-way ANOVA.**

2. To recognize when it is appropriate to apply this test.

Background

Problem Posing

Somatic embryogenesis is the process of embryo initiation and development (Schaeffer, 1990). Embryogenesis from callus growth has been obtained from many plant species (Evans et al., 1981). In addition to rapid propagation, it is hoped that this process will be useful for production of artificial seeds (Wenzel, 1992). Ammirato (1983) developed a standard procedure for somatic embryogenesis and plantlet regeneration of carrot *(Daucus carota).* Disinfected explants of carrot roots are placed on an agar medium containing 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) to initiate callus. The callus is subcultured into liquid media containing 2,4-D to form a suspension culture. Following this, subculturing the suspension cells to liquid media devoid of auxin and other growth regulators allows embryo development in about 8 to 15 days. Plantlets develop on agar media devoid of 2,4-D. Some embryogenesis protocols use only solid media (Kamada & Harada, 1979).

A number of factors, such as growth regulators, sugars, and nitrogen sources, can affect carrot somatic embryogenesis (Ammirato, 1986), thereby affecting the yield of embryos. For example, Masuda et al. (1981), using a modified Murashige and Skoog medium, found that addition of 0.6 to 2.0 g/L casein hydrolysate increased the number of embryos from carrot hypocotyl segments up to 160%. Wochok and Wetherell (1972) tested several concentrations of kinetin and 2,4-dichlorophenoxyacetic acid (2,4-D) and determined that 10^{-7} M kinetin and 10^{-6} M 2,4-D, alone or in combination, could stimulate embryogenesis in older carrot suspension cultures. This is important since the potential for morphogenesis may be lost in older cultures (Ammirato, 1986). Kamada and Harada (1979), using carrot hypocotyl segments, found that organogenesis progressively decreased but embryogenesis progressively increased following 1 to 14 days incubation on callus induction medium. The **general problem** for this lab, then, is to select a protocol that will allow production of a high number of embryos for carrot taproot cultures. Since the number of factors influencing embryogenesis is large, one approach would be to use an experimental design that would allow testing of one of these factors at several levels, using a one-way analysis of variance (ANOVA) for statistical analysis.

Problem Solving

Using a one-way ANOVA aids in determining if any observed differences in sample means are due to the effect of the chosen factor on embryogenesis or if they are due simply to sampling variability. The advantage of the one-way ANOVA over a two-sample experiment (such as in Exercise 2) is that one can not only tell if a particular factor has an effect but can also tell if there is a relationship between concentration of the factor and number of embryos produced (Schmidt, 1979).

The Specific Question

Following initiation of carrot callus on Murashige and Skoog medium, Masuda et al. (1981) tested the influence of casein hydrolysate at several concentrations (0 to 2.0 g/L) on embryogenesis of suspension cultures prepared from carrot hypocotyl segments. Their test medium was Murashige and Skoog medium with the NH_4NO_3 and KNO_3 concentrations modified to 10.3 mM and 24.7 mM, respectively. Since Gamborg's B-5 medium contains KNO_3 at 24.7 mM but NH_4NO_3 only at 1.01 mM, a **specific question** posed by Smith et al. (unpublished) was, "Would casein hydrolysate at 1, 0.5,

1.0, or 2.0 g/L influence embryogenesis of carrot taproot cultures grown on Gamborg's B-5 medium?"

The Hypotheses

For statistical analysis, null and alternative hypotheses are stated. The **null hypothesis** for this experiment is, "The mean number of embryos is the same at all levels of casein hydrolysate." The **alternative hypothesis** is "The mean number of embryos will not be the same at all levels of casein hydrolysate."

The Experimental Design

1. The callus initiation medium was Murashige and Skoog salt base containing 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 100 mg/L inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 0.4 mg/L thiamine HCl, 2 mg/L glycine, 1 g/L casein hydrolysate, 30 g/L sucrose, and 8 g/L agar. Eight milliliters were dispensed into each 20 mm x 150 mm culture tube and sterilized by autoclaving.

2. Carrots *(Daucus carota)* were scrubbed under running tapwater to remove residual soil.

3. The skin was removed, and the carrot was cut transversely into slices about 15-mm thick.

4. The slices were disinfected for 30 minutes using 20% (v/v) bleach and then rinsed with sterile distilled water.

5. The carrot pieces were cut transversely into slices 2-mm thick, and then explants 5-mm wide were removed from the slices, being sure to include the cambium in each slice.

6. This process was repeated to give a total of 40 explants.

7. One explant was transferred to each tube of callus initiation medium and incubated, without light, at 25° C for five weeks.

8. Following five weeks incubation, the explants were subcultured on callus initiation medium for two additional five-week periods, for a total of 15 weeks on callus initiation medium. Then, 10 explants were tested at each level of casein hydrolysate. Gamborg's B-5 medium supplemented with 30 g/L sucrose and 8 g/L agar was used as the test medium. No hormone was added, and the concentration of casein hydrolysate was varied at 0, 0.5, 1.0, and 2.0 g/L.

9. Test cultures were incubated at 25° C with a 16-

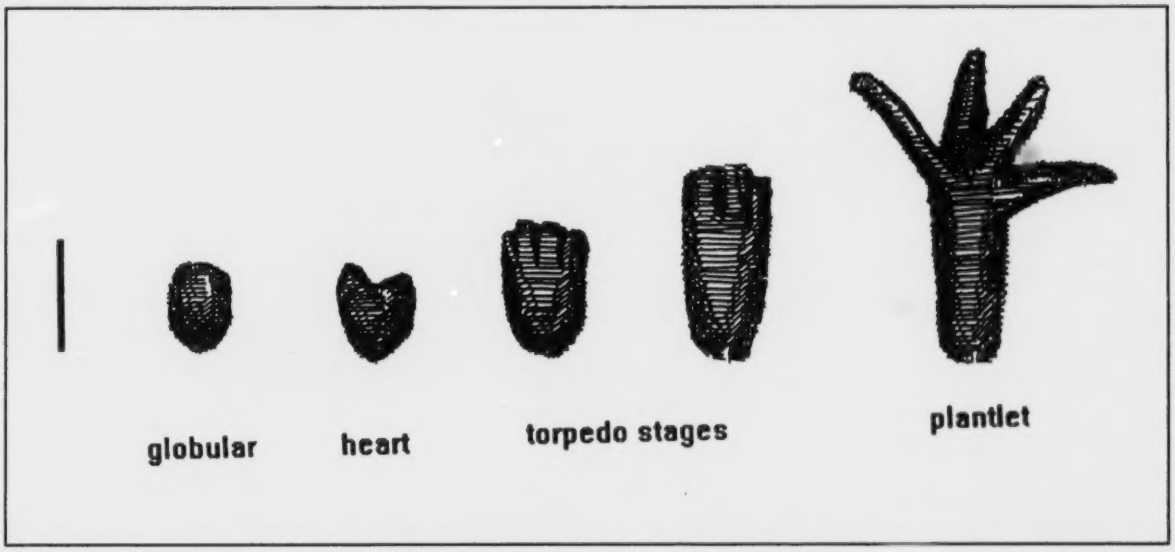

Figure 3.1. Carrot somatic embryos grown after six weeks of culture. A wide range of stages will be visible from globular to plantlet. (Bar = 1 mm).

hour photoperiod (approximately 2,000 lux) for three weeks, and then each explant was subcultured to identical media for an additional three weeks. Following incubation, the number of embryos attaining at least the heart stage of development (Figure 3.1) for each explant at each level of casein hydrolysate was counted.

The Results

After six weeks of incubation, Smith et al. (unpublished) collected the data presented in Table 3.1 on the opposite page.

References

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Zar, J.H. (1996). *Biostatistical Analysis* (3rd ed.). Upper Saddle River, NJ: Prentice Hall, Inc.

Procedure

1. Examine the data presented in Table 3.1 and answer Question 1 on the result sheet.

2. Now analyze the data using the formulas presented in Figure 3.1. Record all calculations on the result sheet.

   - First, compute the between group variation (SS_{bg}), the within group variation (SS_{wg}), and the total variation (SS_{total}).

   - Second, compute the between group mean square (MS_{bg}) and the within group mean square (MS_{wg}).

   - Now compute $F_{observed}$ and compare this to the $F_{critical}$ value at the 0.05 level of significance (see Appendix D). The degrees of freedom for the numerator of $F_{critical}$ is k-1, where k = number of treatments. The degrees of freedom for the denominator $F_{critical}$ is N-k, where N = total number of observations. If this value does not appear in the table, use the critical value with the next lower *df*. (The critical value of F at other levels of significance may be obtained from tables presented in texts of statistical analysis (e.g., Zar, 1996).

- If the observed value of F is less than the critical value of F, accept the null hypothesis.
- If the observed value of F is equal to or greater than the critical value of F, accept the alternative hypothesis.

3. Compare the conclusion following statistical analysis with the one made initially.

Replicate #	Number of Embryos per Explant at Each Level of Casein Hydrolysate (g/L)			
	0.0	0.5	1.0	2.0
1	1	21	169	72
2	31	7	110	60
3	2	1	131	47
4	60	11	127	112
5	8	21	166	98
6	28	35	156	165
7	14	13	97	87
8	2	18	179	88
9	30	13	90	14
10	24	89	129	109
Mean	20	22.9	135.4	85.2

Table 3.1. Number of embryos per explant at each level of casein hydrolysate.

Variation Between Groups

$$SS_{bg} = \frac{\Sigma\,[\,(\Sigma x_{1i})^2 + (\Sigma x_{2i})^2 + (\Sigma x_{3i})^2\,] - (\Sigma x_i)^2}{n} \quad \frac{}{kn}$$

where: x_{1i}, x_{2i}, and x_{3i} are group totals (the sum of all individual values for groups 1, 2, and 3, respectively.)

x_i = individual values
n = number of data for each group
k = number of groups
$df = k\text{-}1$

Variation Within Groups

$$SS_{wg} = \left[\Sigma x_{1i}^2 - \frac{(\Sigma x_{1i})^2}{n}\right] + \left[\Sigma x_{2i}^2 - \frac{(\Sigma x_{2i})^2}{n}\right] + \left[\Sigma x_{3i}^2 - \frac{(\Sigma x_{3i})^2}{n}\right]$$

where: $df = k(n\text{-}1)$

Total Variation

$$SS_{total} = \Sigma x_i^2 - \frac{(\Sigma x_i)^2}{kn}$$

where: $df = kn\text{-}1$

Mean Squares

$$MS_{bg} = \frac{SS_{bg}}{df_{bg}} \qquad MS_{wg} = \frac{SS_{wg}}{df_{wg}}$$

$F_{observed}$

$$F_{observed} = \frac{MS_{bg}}{MS_{wg}}$$

Figure 3.2. Computational formulas for single-factor ANOVA (based upon Schmidt, 1979).

Report 3
Problem Solving Case Study
One-Way Analysis of Variance

Name:

1. Before performing the statistical analysis, does it appear that casein hydrolysate has an influence on the number of embryos produced?

2a. Record the calculations for the between group variation (SS_{bg}), the within group variation (SS_{wg}), and the total variation (SS_{total}).

2b. Record the calculations for the between group mean square (MS_{bg}) and the within group mean square (MS_{wg}).

2c. Record the calculations for $F_{observed}$ and compare this to the $F_{critical}$ value at the 0.05 level of significance (see Appendix D).

2d. Should the null hypothesis or the alternative hypothesis be accepted?

3. Compare the conclusion following statistical analysis with the one made initially.

4. Does it appear that all treatment levels have an effect or is one level significantly better? How can you tell? (See Exercise 5).

* *Refer to Appendix C to compare your analysis and conclusions with those of the original authors.*

4. Problem Solving Case Study: Two-Way Analysis of Variance

Objectives

1. To gain practice using a specific problem-solving approach known as the **two-way ANOVA**.

2. To recognize when it is appropriate to apply this test.

Background

Problem Posing

Shoot tip cultures are analogous to small cuttings (Steward, 1983). They consist of the apical meristem plus one to several primordial leaves (Schaeffer, 1990). Due to their genetic stability, they are used for rapid asexual propagation of many economically important plants (Hu & Wang, 1983; Rice, 1992). Applications of shoot tip culturing include production of metabolites in culture (Heble, 1985) and germplasm preservation (Kartha, 1985; Kartha, 1987).

A **general problem** for these applications, however, is to develop a reliable protocol for shoot tip manipulation and regeneration of plants for each variety of plant. For example, differences in shoot regeneration were observed for 16 genotypes of *Populus deltoides* (Coleman & Ernst, 1989). One of the factors important for developing a successful protocol is exogenous growth regulator supply. Cytokinins and auxins are the major growth regulators used for shoot tip cultures, and they may be used in combination or alone (Hu & Wang, 1983). One approach to this problem is to use an experimental design that would allow analysis of data using a **two-way analysis of variance (ANOVA)**.

Problem Solving

A two-way factorial experimental design allows determination of the effects of each factor, alone and in combination (Schmidt, 1979; Zar, 1996). The ANOVA will determine the significance of the effect of the auxin, the effect of the cytokinin, and any difference in the effect of auxin resulting from changes in the cytokinin concentration.

The Specific Question

Griga et al. (1984) determined that 20 µM benzylaminopurine (BA) combined with 0.1 µM naphthaleneacetic acid (NAA) gave the highest rate of shoot proliferation for *Pisum sativum* L. cv Bohatyr. Smith et al. (1997) asked the the **specific question**: What is the effect of BA and NAA, alone and in combination, on proliferation of *Pisum sativum* L. cv Little Marvel shoot tips?

The Hypotheses

The **null hypotheses** being tested for this experiment are:

1. There is no effect of BA treatment on the average number of pea shoot tips produced.

2. There is no effect of NAA treatment on the average number of pea shoot tips produced.

3. There is no interaction of BA and NAA treatment on the average number of pea shoot tips produced.

The **alternative hypotheses** are:

1. There is an effect of BA treatment on the average number of pea shoot tips produced.

2. There is an effect of NAA treatment on the average number of shoot tips produced.

3. There is an interaction of BA and NAA treatment on the average number of pea shoot tips produced.

The Experimental Design

1. The medium consisted of Murashige and Skoog salt base (available at biological supply companies)

with 1 mg/L thiamine HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 100 mg/L inositol, 30 g/L sucrose, and 8 g/L agar.

2. The hormone concentration was adjusted to obtain the six combinations used in the experimental design (Table 4.1). The medium was sterilized by autoclaving, and five milliliters were dispensed into separate sterile 16 mm x 100 mm tubes.

3. Pea seeds (*Pisum sativum* L. Little Marvel) were disinfected by soaking them in 95% ethanol for one minute followed by 20 minutes in 20% (v/v) bleach. Then the seeds were rinsed three times with sterile distilled water.

4. The seeds were incubated in a sterile beaker containing distilled water at 25° C for five to seven days.

5. Following incubation, the terminal 5 to 6 mm of shoot tip was dissected from each seedling.

6. Each shoot tip was transferred to one of the treatment tubes until each treatment was replicated eight times.

7. All treatment tubes were incubated at 26° C with a 16-hour photoperiod (approximately 2,000 lux) for six weeks.

The Results

The total number of shoots forming per explant (Figure 4.1) in response to BA and NAA concentrations is presented in Table 4.1.

References

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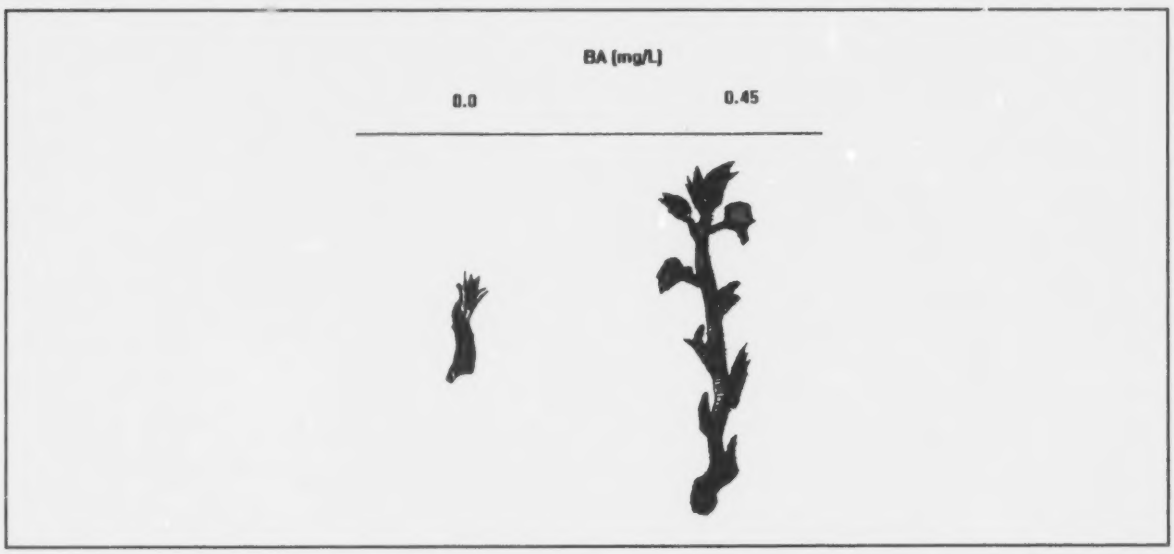

Figure 4.1. Appearance of shoots in response to BA concentration. The shoot on the left represents those grown for six weeks at 0 mg/L BA. They are 10 mm in length and retain the original three buds. The shoot on the right represents those grown at 0.45 mg/L. They are 20 mm in length and developed from one to three buds in each axil.

Hu, C.Y. & Wang, P.J. (1983). Meristem, shoot tip, and bud cultures. In D.A. Evans, W.R. Sharp, P.V. Ammirato & Y. Yamada (Eds.), *Handbook of Plant Cell Culture, Volume I,* 177-227. New York: Macmillan Publishing Company.

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Zar, J.H. (1996). *Biostatistical Analysis* (3rd ed.). Upper Saddle River, NJ: Prentice Hall, Inc.

Procedure

1. Examine the data presented in Table 4.1 and answer Question 1 on the result sheet.

2. Now, analyze the data using the formulas presented in Figures 4.2a and 4.2b. Record all calculations on the result sheet.

   - First, compute the between column variation (SS_c), the between row variation (SS_r), the between rows and columns variation (SS_{rxc}), the within groups variation (SS_{wg}), and the total variation (SS_{total}).

   - Second, compute the row mean square (MS_r), the column mean square (MS_c), the within group mean square (MS_{wg}), and the between rows and columns mean square (MS_{rxc}).

   - Now compute $F_{observed}$ values for factor 1 main effect, for factor 2 main effect, and for factor 1 x factor 2 interaction and compare this to the $F_{critical}$ values at the 0.05 level of significance (see Appendix D), using the corresponding row, column, and interaction *df* for the numerator variance and the within group *df* for the denominator variance. If these values do not appear in the table, use the critical values with the next lower *df*. (The critical values of *F* at other levels of significance may be obtained from tables presented in texts of statistical analysis; e.g., Zar, 1996).

   - If the observed value of *F* is less than the critical value of *F*, accept the null hypothesis. If the observed value of *F* is equal to or greater than the critical value of *F*, reject the null hypothesis.

3. Compare your conclusions following statistical analysis with those made initially.

Table 4.1. The influence of NAA and BA on multiple shoot formation in peas.

NAA mg/L	BA mg/L 0.0	BA mg/L 0.45
0.0	3 3 3 3 3 3 3 3	11 11 10 16 8 11 12 13
0.02	2 3 3 3 3 5 3 3	15 19 12 10 17 17 15 14
0.20	3 3 3 3 3 3 3 3	15 15 10 12 12 13 13 10
	$\bar{X} = 3.04$	$\bar{X} = 12.96$

Variation Between Columns

$$SS_c = \frac{\Sigma [(\Sigma x_{c1i})^2 + (\Sigma x_{c2i})^2]}{nk} - \frac{(\Sigma x_i)^2}{nkm}$$

where: x_{c1i} and x_{c2i} are column totals (the sum of all individual values for columns 1 and 2, respectively).
x_i = individual values
n = number of data for each cell
k = number of rows (levels of factor 1)
m = number of columns (levels of factor 2)
$df = m\text{-}1$

Variation Between Rows

$$SS_r = \frac{\Sigma [(\Sigma x_{r1i})^2 + (\Sigma x_{r2i})^2 + (\Sigma x_{r3i})^2]}{nm} - \frac{(\Sigma x_i)^2}{nkm}$$

where: x_{r1i}, x_{r2i}, and x_{r3i} are row totals (the sum of all individual values for rows 1, 2, and 3, respectively).
$df = k\text{-}1$

Variation Between Rows and Columns

$$SS_{rxc} = \frac{\Sigma [(\Sigma x_{r1c1i})^2 + (\Sigma x_{r1c2i})^2 + \ldots + (\Sigma x_{r3c1i})^2 + (\Sigma x_{r3c2i})^2]}{n} - \frac{\Sigma [(\Sigma x_{r1i})^2 + (\Sigma x_{r2i})^2 + (\Sigma x_{r3i})^2]}{nm} - \frac{\Sigma [(\Sigma x_{c1i})^2 + (\Sigma x_{c2i})^2]}{nk} + \frac{(\Sigma x_i)^2}{nkm}$$

where: $x_{r1c1i} \ldots x_{r3c2i}$ are cell totals (the sum of all individual values for each cell.)

Figure 4.2a. Computational formulas for two-factor ANOVA (based upon Schmidt, 1979).

Variation Within Groups

$$SS_{wg} = \left[\sum x_{r1c1i}{}^2 - \frac{(\sum x_{r1c1i})^2}{n} \right] + \left[\sum x_{r1c2i}{}^2 - \frac{(\sum x_{r1c2i})^2}{n} \right]$$

$$+ \left[\sum x_{r2c1i}{}^2 - \frac{(\sum x_{r2c1i})^2}{n} \right] + \left[\sum x_{r2c2i}{}^2 - \frac{(\sum x_{r2c2i})^2}{n} \right]$$

$$+ \left[\sum x_{r3c1i}{}^2 - \frac{(\sum x_{r3c1i})^2}{n} \right] + \left[\sum x_{r3c2i}{}^2 - \frac{(\sum x_{r3c2i})^2}{n} \right]$$

where: $df = km\ (n\text{-}1)$

Total Variation

$$SS_{total} = \sum x_i{}^2 - \frac{(\sum x_i)^2}{nkm}$$

where: $df = nkm\text{-}1$

Mean Squares

$$MS_r = \frac{SS_r}{df_r} \qquad MS_{wg} = \frac{SS_{wg}}{df_{wg}}$$

$$MS_c = \frac{SS_c}{df_c} \qquad MS_{rxc} = \frac{SS_{rxc}}{df_{rxc}}$$

For Factor 1 Main Effect

$$F_{observed} = \frac{MS_r}{MS_{wg}}$$

For Factor 1 x Factor 2 Interaction

$$F_{observed} = \frac{MS_{rxc}}{MS_{wg}}$$

For Factor 2 Main Effect

$$F_{observed} = \frac{M_c}{MS_{wg}}$$

Figure 4.2b. Computational formulas for two-factor ANOVA (based upon Schmidt, 1979).

Report 4
Problem Solving Case Study
Two-Way Analysis of Variance Name:

1. Before performing the statistical analysis, which treatments appear to be most effective, if any?

2a. Record the calculations for the between column variation (SS_c), the between row variation (SS_r), the between rows and columns variation (SS_{rxc}), the within groups variation (SS_{wg}), and the total variation (SS_{total}).

2b. Record the calculations for the row mean square (MS_r), the column mean square (MS_c), the within group mean square (MS_{wg}), and the between rows and columns mean square (MS_{rxc}).

2c. Record the calculations for $F_{observed}$ values for factor 1 main effect, for factor 2 main effect, and for factor 1 x factor 2 interaction and compare this to the $F_{critical}$ values at the 0.05 level of significance (see Appendix D).

2d. Should the null hypothesis or the alternative hypothesis be accepted?

3. Compare the conclusions following statistical analysis with the ones made initially.

** Refer to Appendix C to compare your analysis and conclusions with those of the original authors.*

5. The Tukey Test

Objectives

1. To gain practice using a specific multiple comparision technique known as the **Tukey test**.

2. To recognize when it is appropriate to apply this test.

Background

Rejection of the null hypothesis for a one-way (single factor) or a two-way factorial analysis of variance does not imply that all means are different from one another. To determine this, a multiple comparison procedure, such as the *Tukey test,* is used to decide which pairs of means are significantly different (Zar, 1996). The formulas and procedure for this test for one-way and two-way ANOVAs are presented in Figures 5.1 and 5.2, respectively. In Exercise 3, the null hypothesis is rejected, and thus a multiple comparison procedure must be performed. The computations for this procedure, using the data from Exercise 3, are presented in Table 5.1. In Exercise 4, all three alternative hypotheses are accepted. Since there were only two levels of BA, the Tukey test is not necessary; however, there are more than two levels of NAA, and thus the Tukey test must be performed.

Reference

Zar, J.H. (1996). *Biostatistical Analysis* (3rd ed.). Upper Saddle River, NJ: Prentice Hall, Inc.

Procedure

1. Using the procedure and formulas in Figure 5.1, calculate q values and compare your answers with those in Table 5.1.

2. The critical value of q ($q_{alpha,v,k}$), where v = the within group *df* and k = the number of means being tested, is obtained from a table (see Appendix D). Since $q_{0.05,36,4}$ does not appear in the table, the critical value with the next lower df is used. If the observed or calculated q value is equal to or greater than $q_{critical}$, then the null hypothesis is rejected.

   In this case, the means for the 2.0 and 1.0 g/L casein hydrolysate are different from each other and from the 0.5 and 0.0 g/L groups; however, the means for the 0.5 and 0.0 g/L groups are not different. That is, addition of 0.5 g/L casein hydrolysate is of no benefit; whereas, increasing the concentration to 1.0 g/L increases the number of embryos significantly. Increasing the concentration to 2.0 g/L causes a decrease in the number of embryos.

3. Now, using the procedure and formulas in Figure 5.2, calculate the *q values* for the comparison of sample means from Exercise 4. Record your calculations in the table provided.

4. Determine the critical value of q and answer the questions.

1. Assign a number to all sample means:

sample	0.0 g/L CH	0.5 g/L CH	1.0 g/L CH	2.0 g/L CH
means numbered	1	2	3	4
sample means	20	22.9	135.4	85.2

2. Arrange sample means in order of increasing magnitude:

means ranked	20	22.9	85.2	135.4
sample number	1	2	4	3

3. Tabulate pairwise differences by comparing the largest mean against the smallest and then the largest mean against the next smallest. Then compare the second largest mean to the smallest (see Table 5.1).

4. Then a *q value* is calculated by dividing the difference between these means by the appropriate standard error:

$$SE = \sqrt{\frac{MS_{wg}}{n}} \qquad q = \frac{\overline{X}_B - \overline{X}_A}{SE}$$

where:

MS_{wg} = the within groups mean square.
n = number of data in each of groups A and B.

5. Compare the calculated *q value* to the critical *q value*.

Figure 5.1. Procedure for Tukey multiple comparison test used with a one-way ANOVA (Zar, 1996).

1. Assign a number to all sample means from Exercise 4.

2. Arrange sample means in order of increasing magnitude.

3. Tabulate pairwise differences by comparing the largest mean against the smallest and then the largest mean against the next smallest. Then compare the second largest mean to the smallest.

4. Calculate a *q value* by dividing the difference between these means by the appropriate standard error:

If factor 1 differences are significant:

$$SE = \sqrt{\frac{MS_{wg}}{nm}} \qquad q = \frac{\overline{X}_B - \overline{X}_A}{SE}$$

If factor 2 differences are significant:

$$SE = \sqrt{\frac{MS_{wg}}{nk}} \qquad q = \frac{\overline{X}_B - \overline{X}_A}{SE}$$

where:

MS_{wg} = the within groups mean square.
n = number of data in each cell
m = number of columns (levels of factor 2)
k = number of rows (levels of factor 1)

5. Compare the calculated *q value* to the critical *q value*.

Figure 5.2. Procedure for Tukey multiple comparison test used with a two-way ANOVA (Zar, 1996).

Table 5.1. Tukey multiple comparison test for casein hydrolysate concentration. Alpha = 0.05.

Comparison (B vs. A)	Difference $(\bar{X}_B - \bar{X}_A)$	*SE*	*q*	$q_{0.05,30,4}$	Conclusion
3 vs. 1	115.4	9.5124	12.1315	3.845	reject
3 vs. 2	112.5	9.5124	11.8267	3.845	reject
3 vs. 4	50.2	9.5124	5.2773	3.845	reject
4 vs. 1	65.2	9.5124	6.8542	3.845	reject
4 vs. 2	62.3	9.5124	6.5493	3.845	reject
2 vs. 1	2.9	9.5124	0.3049	3.845	accept

Report 5
The Tukey Test

Name:

1. Prior to performing the Tukey test for the sample means from Exercise 4, did any means appear to be significantly different? Explain.

2. Assign a number to all sample means from Exercise 4:

sample	0.0 mg/L NAA	0.02 mg/l NAA	0.20 mg/L NAA
means numbered	1	2	3
sample means			

3. Arrange sample means in order of increasing magnitude.

means ranked

sample number

4. Tabulate pairwise differences and a *q value*. Determine and record the critical value of q.

Comparison (B vs. A)	Difference $(\overline{X}_B - \overline{X}_A)$	*SE*	q	$q_{0.05,v,k}$	Conclusion
__ vs. __	—	—	—	—	—
__ vs. __	—	—	—	—	—
__ vs. __	—	—	—	—	—

(continued on next page)

5. Compare the conclusions following the Tukey test with the ones made initially.

** Refer to Appendix C to compare your analysis and conclusions with those of the original authors.*

6. Presentation

Objectives

1. To gain practice analyzing the basic parts of a **poster presentation**.

2. To gain practice using this form of presentation.

Background

The experiments presented in the following exercises were developed to provide specific opportunities for problem posing, experimental design, and analysis and interpretation of data. This gives students a chance to explore more thoroughly material presented in lecture. Problem posing is a powerful vehicle for teaching the content of the domain along with its working procedures (Peterson & Jungck, 1988); however, it does take practice to do this (Seago, 1992). Still, this may be a useful approach since there is some evidence that learning is a matter of both behavioral patterning by reinforcement (behaviorism) and the storage and use of knowledge (cognitivism) (Petri & Mishkin, 1994).

Once data have been collected and analyzed, the findings must be presented. Students have not done science until they have reported their results and convinced their peer group as to the reasonableness of their hypotheses (Peterson & Jungck, 1988). The process of presentation is complex, however, especially for the beginning student. There are many parts to the process, and these will vary depending upon the type of presentation. In this exercise, a sample *poster presentation* will be discussed.

Posters can be set up in a variety of formats, including a format similar to a scientific paper, which usually includes the following parts: **title, abstract, introduction, materials and methods, results, discussion**, and **references cited**. A major difference between a poster and a paper is that poster presentations use large print to allow reading from a distance. Also, the poster presentation must fit within a specified area, such as 4 ft. x 4 ft. or 4 ft. x 8 ft.; thus, each of these parts is abbreviated to fit within one or two pages. The presenter usually is assigned a time to stand by his or her poster to provide additional explanation.

A sample poster, presented at the National Association of Biology Teachers 1994 National Convention, is provided at the end of this exercise. The project was developed to allow a student to explore the use of tissue culture as a means of producing secondary metabolites (see Exercise 23).

Sample Poster Format

The **title** of the poster generally stands alone and should function as a label. It should identify the contents clearly but be as brief as possible. The print is very large, about three-fourths to one-inch (¾" to 1") high — about 80 point size type.

An **abstract** follows the title. The heading for the abstract and all subsequent headings should be centered and have print larger than the text but smaller than the title, about one-third to one-half inch (⅓" to ½") high — 40 point size type. Other items sometimes included as part of the abstract are: the **title** and **authors' names** and the **department** and **institutional affiliation**. This makes it convenient for use as a handout. The authors and affiliation also may be printed on a page separate from the abstract. Some conferences may ask that names of student authors be underlined or otherwise marked.

The text of the abstract and all subsequent text should have print about three-sixteenths to one-quarter inch (3/16" to ¼") high — about 22 point size type — and be single spaced. Some conferences may request that text be full or left justified. Text for the abstract includes one to a few sentences for each of the following: the **purpose** for the study, the **methods** used, the **results** obtained, and the **conclusions** reached.

In the **introduction**, review what information is already

known about the problem being studied. Also in this section, the purpose of the study should be stated. All sources of information reviewed must be cited. Citation within the text includes name(s) of author(s) and year of publication.

The **material and methods** in scientific papers are described in sufficient detail to allow others to repeat the work. If the poster presentation is confined to a small area, this may not be possible, and more detailed explanation will be required orally. Mention statistical procedures, if any, used in the analysis of results.

The **results** are presented in graphs, tables, or illustrations. Keep them simple for poster presentations. Each figure or table must be cited and described in the text of this section. Include results of all statistical analyses.

In the **discussion** section, there should be one to several statements about the biological conclusions that can be drawn from the results. Compare your results to the findings of similar studies mentioned in the introduction. Are there any generalizations that can be made from this comparison?

In the **references** section, list only references cited in the text. List references in alphabetical order. The format for this section varies. One commonly used format for journal articles is: author(s), year of publication, title of paper, journal title underlined, volume, and pages. For books, a common format used is: author(s), year of publication, title of chapter, editor(s), title of book underlined, pages, place of publication, and name of publisher.

For the **presentation**, it is convenient to attach each section of the poster to individual pieces of poster board. For example, when using standard size paper (8.5 x 11 inches), prepare individual background poster pieces 9 x 12 inches; then attach the typed pages to the individual poster pieces using glue or tape (such as Scotch® wallsaver poster tape). This is best done a few days prior to the presentation to maintain the fresh appearance of the poster.

At the presentation, the individual sections will have to be attached to a display board using push pins. Bring extra pins, poster tape, Wite Out®, and copies of the poster in case of problems. The sections may be attached to the poster board starting in the upper-left corner, working down in a column, and then continuing from left to right (Figure 6.1).

References

Peterson, N.S. & Jungck, J.R. (1988). Problem posing, problem solving and persuasion in biology. *Academic Computing, 2,* 14-17; 48-50.

Petri, H.L. & Mishkin, M. (1994). Behaviorism, cognitivism and the neuropsychology of memory. *American Scientist, 82,* 30-37.

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Additional References

The following references will be helpful with different aspects of the presentation process:

Collins, B.K. (1986). Photography in the classroom. *The American Biology Teacher, 48,* 489-491.

Cothron, J.H., Giese, R.N. & Rezba, R.J. (1989). Writing results & conclusions. *The American Biology Teacher, 51,* 239-242.

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Germann, P.J. (1991). Developing science process skills through directed inquiry. *The American Biology Teacher, 53,* 243-247.

Greene, P.E. (1989). Preparation of scientific papers. *The American Biology Teacher, 51,* 438-439.

Kuyper, B.J. (1991). Bringing up scientists in the art of critiquing research. *Bioscience, 41,* 248-250.

Labov, J.B. & Firmage, D.H. (1994). Introducing con-

cepts of random ordering & random assignment of subjects: Computer-assisted classroom & laboratory exercises. *The American Biology Teacher, 56,* 169-173.

Pechenik, J.A. & Tashiro, J.S. (1992). The graphing detective: An exercise in critical reading, experimental design & data analysis. *The American Biology Teacher, 54,* 432-435.

Schlenker, R.M. (1990). Student research report writing. *The American Biology Teacher, 52,* 491-492.

Ward, S. (1983). A research seminar for high school students. *The American Biology Teacher, 45,* 383-386.

Procedure

1. Examine the sample poster that follows and evaluate it using the questions provided on the report sheet as a guide.
2. Using the guidelines and examples for problem posing, problem solving, and presentation provided in this unit, your instructor may ask you to design an investigation of your own for presentation.

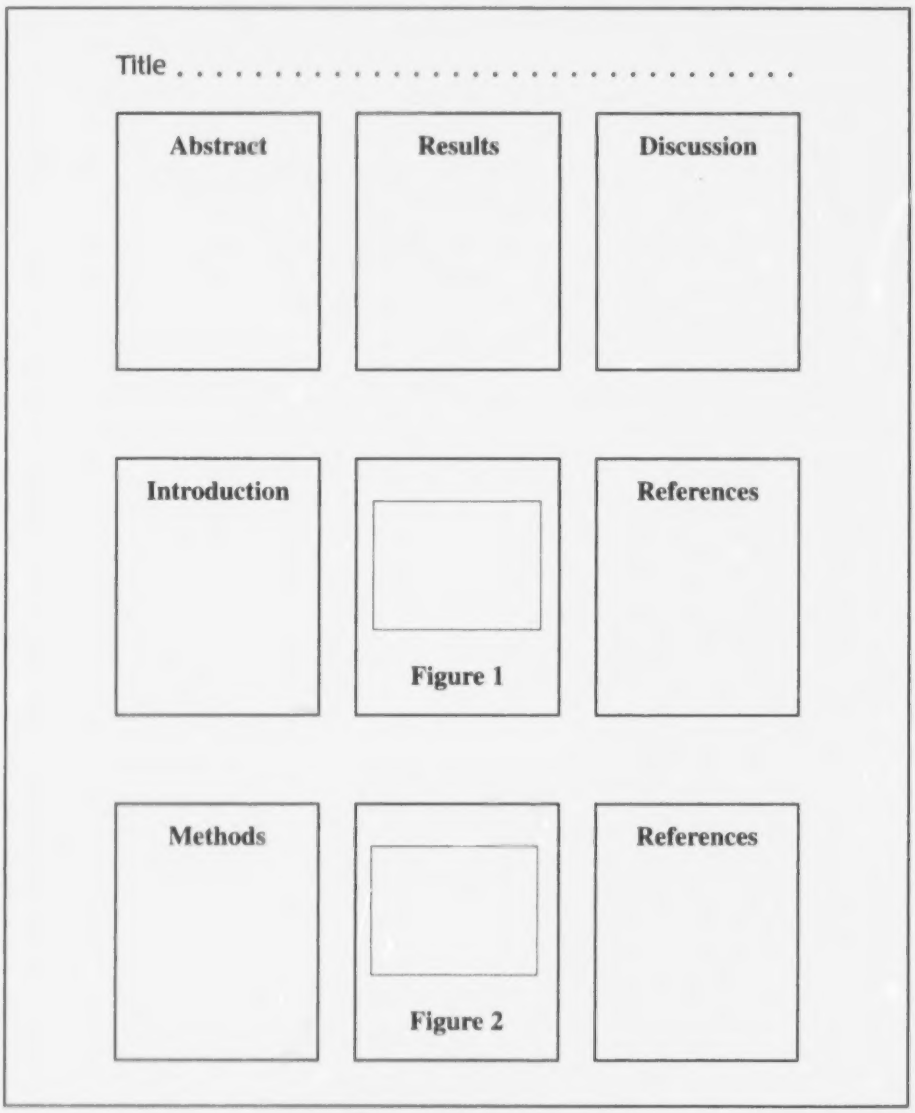

Figure 6.1. Sample poster setup.

Sample Poster Presentation

The following poster was presented at the National Association of Biology Teachers' 1994 National Convention. The style is similar to that of a paper presentation, with full sentences and paragraphs. The title with one-inch-high letters has been omitted for convenience. The abstract page includes the title, authors, department, and affiliation, since this sheet was also used as a handout. The text is full justified. To save space here, the text type size has been reduced. The previous guidelines as to type size should be followed in actual poster presentations for easier readablility from a distance.

Influence of Mercuric Chloride on Furano-Terpene Production of Sweet Potato Root Cell Suspension Cultures

Scott D. Drummond (John R. Porter, Robert A. Smith), Department of Biology, Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104

In intact root tissue, furano-terpenes are produced in response to stressors such as mercuric chloride. In this study, cell suspension cultures of sweet potato (*Ipomoea batatas* (L.) Lam. cv Centennial) roots were cultured in Gamborg's B-5 medium containing 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 20 g/L sucrose. Cultures were treated with 0.0, 0.01, 0.1, 1.0, and 10.0 mg/L $HgCl_2$. Furano-terpene production was maximum with 1.0 mg/L $HgCl_2$. These results provide evidence that furano-terpenes can be stimulated using $HgCl_2$ as a stressor in cell suspension cultures.

INTRODUCTION

Sweet potatoes *(Ipomoea batatas)* belong to the morning glory family, the Convolvulaceae. Efforts are being made to improve sweet potatoes from many aspects, including yield, disease and insect resistance, and nutritional quality (Henderson et al., 1984). In addition, there is an effort to develop industrial uses for sweet potatoes, such as gasohol, animal feed (Henderson et al., 1984), and the use of sweet potato cell cultures for production of secondary products, such as furano-terpenes (Oba & Uritani, 1979) and ergot alkaloids (Kishor & Mehta, 1987).

Oba and Uritani (1979) found that yeast extract and sucrose, but not $HgCl_2$ or *Ceratocystis fimbriata* spores are important for furano-terpene production in suspension culture. Furano-terpenes can be produced by treatment of whole sweet potato root tissue with *Ceratocystis fimbriata* spores (Inoue & Uritani, 1979) or $HgCl_2$ (Oba et al., 1976). Also, metal ions have been shown to play significant roles in altering the expression of secondary metabolic pathways in plant cell culture (Whitaker & Hashimoto, 1986).

Oba and Uritani (1979) tested $HgCl_2$ at concentrations of 0.01% to 0.1%. These are comparable to levels of macronutrients found in some plant culture media (Murashige & Skoog, 1962; Gamborg et al., 1968). The purpose of our study was to investigate the effect of $HgCl_2$ at micronutrient levels (0.0, 0.01, 0.1, 1.0, 10.0 mg/L) on furano-terpene production in sweet potato root cell cultures.

METHODS

Callus cultures of sweet potato (*Ipomoea batatas* (L.) Lam cv. Centennial) root were initiated on Gamborg's B-5 medium supplemented with 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 20 g/L sucrose, and 8 g/L Difco Bacto agar. The callus tissue was subcultured every 30 days.

Cell suspension cultures were initiated by inoculating 3 g callus tissue into 50 ml liquid Gamborg's B-5 medium. Flasks were incubated at 25° C without light and agitated at 150 rpm. Following this, 14 ml of settled cell volume were used for subculturing. Inoculation size (7 and 14 ml settled cell volume), yeast extract (0 and 5 g/L), and $HgCl_2$ (0.0, 0.01, 0.1, 1.0, 10.0 mg/L) concentrations were varied. Furano-terpene content of inoculated media was analyzed at 0, 1, and 2 days of subculture. Cells were subcultured with fresh medium after each sampling.

Samples were extracted with an equal amount of methanol:chloroform (1:1, v/v). The chloroform phase was separated using Whatman 1PS phase separator paper and allowed to dry overnight at room temperature. Following extraction and drying, samples were dissolved in 1 ml chloroform. Forty µl of each sample were developed on silica-gel TLC plates with benzene:ethylacetate (8:2, v/v) for 15 cm. Terpenes were visualized with 2% p-dimethylaminobenzaldehyde in concentrated HCL:H_2O (1:1, v/v).

RESULTS

The effects of inoculation density and yeast extract concentration are presented in Figure 1. There was a slight increase in furano-terpene production at the higher inoculation density for cultures with and without yeast extract. There was a greater increase in furano-terpene production after 1 and 2 days of subculture with yeast extract.

The effects of $HgCl_2$ concentration on furano-terpene production are presented in Figures 2 and 3. There was a significant increase in furano-terpene production after 1 and 2 days of subculture at 1.0 mg/L $HgCl_2$ concentration. At 10.0 mg/L $HgCl_2$, furano-terpene production was decreased.

DISCUSSION

These experiments were designed to show the effects of inoculation density, yeast extract concentration, and $HgCl_2$ concentration on induction of furano-terpenes in sweet potato cell suspension cultures. Oba and Uritani (1979) tested $HgCl_2$ as an inducer in sweet potato suspension culture at concentrations of 0.01% to 0.1%. No terpenes were produced.

In plant tissue culture media, micronutrients are normally added at concentrations of 0.25 to 10 mg/L. Our results show furano-terpene production at 1 mg/L $HgCl_2$ with decreasing production at 10 mg/L $HgCl_2$. It may be that at 1 mg/L $HgCl_2$, there is modification of enzyme proteins participating in terpene synthesis from latent to active form (Oba et al., 1976); whereas, at high concentration, there is more general protein poisoning.

Increasing inoculation density also increased concentration of furano-terpenes, but only slightly. With yeast extract, there was a significant increase in furano-terpene production. Oba and Uritani (1979) also determined that yeast extract was important for furano-terpene production.

The results of our experiments provide support for the hypothesis that $HgCl_2$ can be used to influence production of terpenes in sweet potato cell suspension cultures.

REFERENCES

Gamborg, O.L., Miller, R.A. & Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research, 50,* 151-158.

Henderson, J.H.M., Phills, B.R. & Whatley, B.T. (1984). Sweet potato. In W.R. Sharp, D.A. Evans, P.V. Ammirato & Y. Yamada (Eds.), *Handbook of Plant Culture: Volume II,* 302-326. New York: Macmillan Publishing Company.

Inoue, H. & Uritani, I. (1979). Biosynthetic correlation of various phytoalexins in sweet potato root tissue infected by *Ceratocystis fimbriata. Plant and Cell Physiology, 20,* 1307-1314.

Kishor, P.B.K. & Mehta, A.R. (1987). Ergot alkaloid production in suspension cultures of *Ipomoea batatas* Poir. *Current Science, 56,* 781-783.

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum, 15,* 473-497.

Oba, K., Tatematsu, H., Yamashita, K. & Uritani, I. (1976). Induction of furanoterpene production and formation of the enzyme system from mevolonate to isopentenyl pyrophosphate in sweet potato root tissue injured by *Ceratocystis fimbriata* and by toxic chemicals. *Plant Physiology, 58,* 51-56.

Oba, K. & Uritani, I. (1979). Biosynthesis of furano-terpenes by sweet potato cell culture. *Plant and Cell Physiology, 20,* 819-826.

Whitaker, R.J. & Hashimoto, T. (1986). Production of secondary metabolites. In D.A. Evans, W.R. Sharp & P.V. Ammirato (Eds.), *Handbook of Plant Cell Culture: Techniques and Applications*, 264-286. New York: MacMillan Publishing Company.

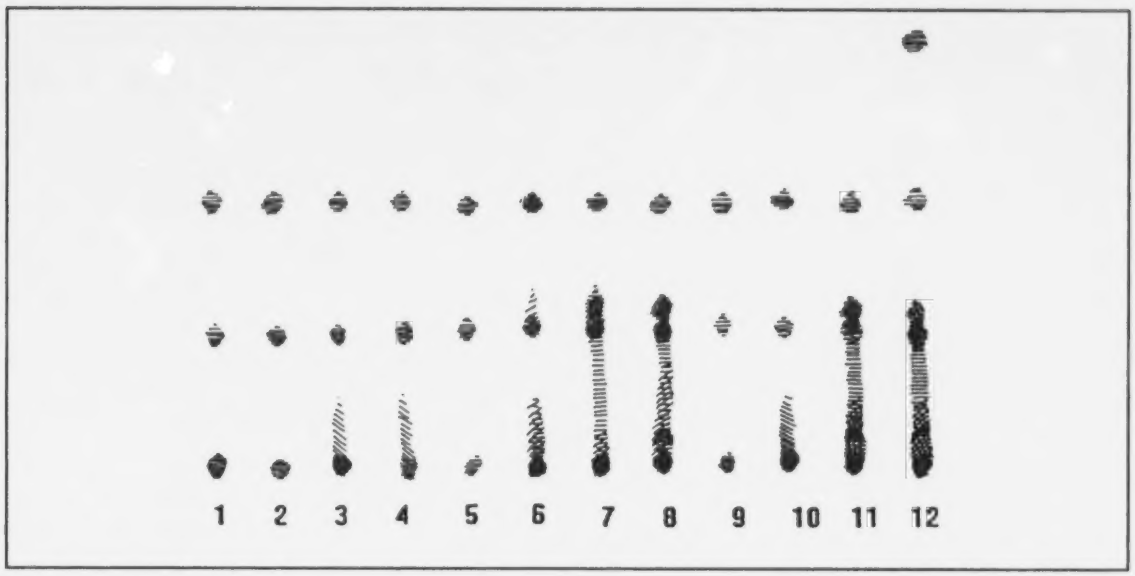

Figure 1. Effects of inoculation density (ID) and yeast extract (YE) on sweet potato cell suspension cultures; (1) 7 ml ID, 0 g YE, day 0: (2) 14 ml ID, 0 g YE, day 0; (3) 7 ml ID, 5 g YE, day 0; (4) 14 ml ID, 5 g YE, day 0; (5) 7 ml ID, 0 g YE, day 1; (6) 14 ml ID, 0 g YE, day 1; (7) 7 ml ID, 5 g YE, day 1; (8) 14 ml ID, 5 g YE, day 1; (9) 7 ml ID, 0 g YE, day 2; (10) 14 ml ID, O g YE, day 2; (11) 7 ml ID, 5 g YE, day 2; (12) 14 ml ID, 5 g YE, day 2.

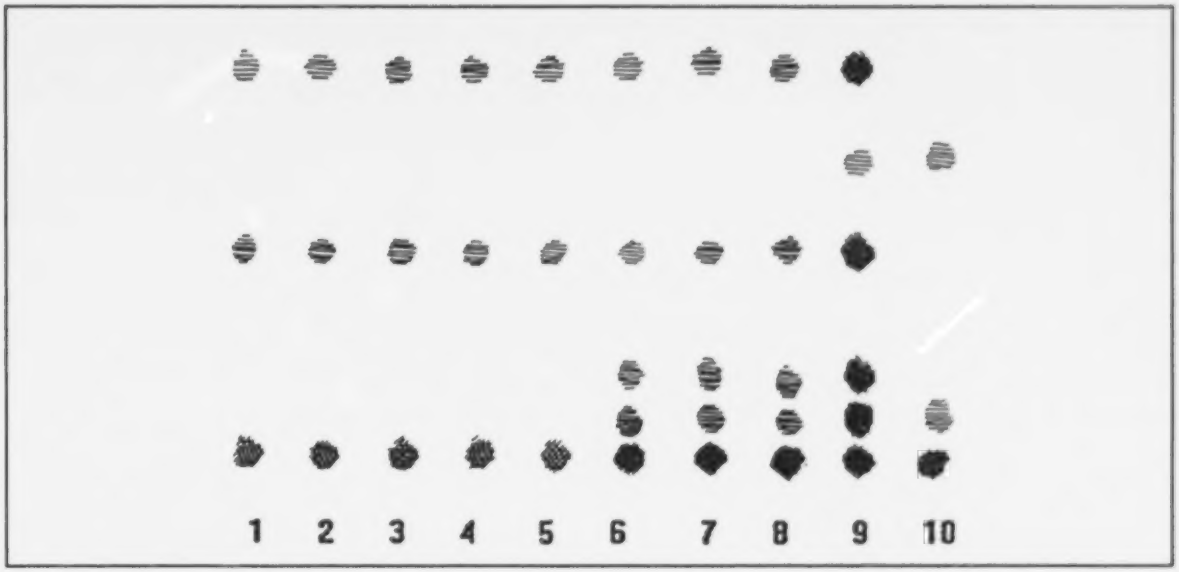

Figure 2. Effect of $HgCl_2$ over a two-day period: (1-5) $HgCl_2$ concentrations of 0, 0.01, 0.1, 1.0, and 10.0 mg/L respectively, at day 0; (6-10) $HgCl_2$ concentrations of 0, 0.01, 0.1, 1.0, and 10.0 mg/L respectively, at day 1.

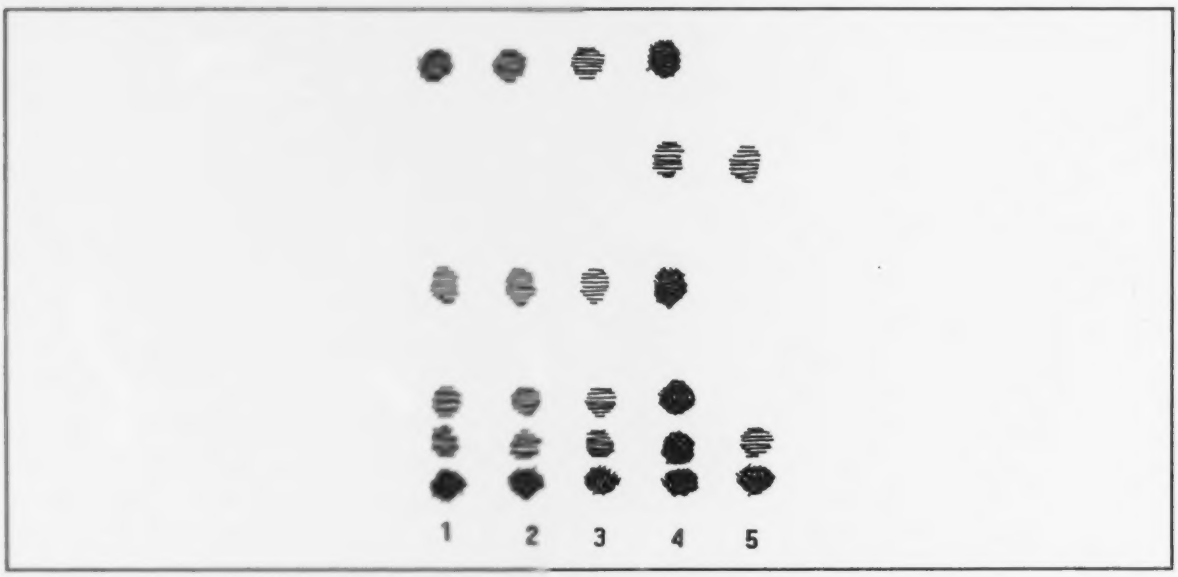

Figure 3. Effect of $HgCl_2$ over a two-day period: (1-5) HgC_2 concentrations of 0, 0.01, 0.1, 1.0, and 10.0 mg/L respectively, at day 2.

Report 6
Presentation Name:

I. Evaluate the sample poster using the questions below as a guide.

(A) **Title**

Does the title function as a label?

(B) **Abstract**

In the abstract section, identify the following statements:

purpose

methods

results

conclusion(s)

(C) **Introduction**

1. In the introduction section, identify the following statements:

what is already known about the problem

purpose of the study

2. Are sources referenced properly (names and dates)?

(D) **Materials and Methods**

1. In the materials and methods sections, are any steps obviously missing?

2. Were any statistical tests used?

(E) **Results**

1. In the results section, are the results presented as graphs, tables, or illustrations?

2. Are the results quantitative or qualitative?

3. Were the results discussed in the text of this section?

4. What were the major trends?

5. Do these trends match those stated in the abstract?

(F) **Discussion**

1. In the discussion section, what conclusions were drawn?

2. How do these conclusions compare with those of similar studies mentioned in the introduction?

(G) **References**

1. In the reference section, are references cited in alphabetical order?

2. Was the correct format used for all book references?

3. Was the correct format used for all journal references?

II. Using the guidelines and examples for problem posing, problem solving, and presentation provided in this unit, design an investigation of your own for presentation. Write your proposal on the form provided in Appendix E.

UNIT II – ASEPTIC TECHNIQUE

Success in culturing plant cells, tissues, and organs is dependent upon an understanding of aseptic technique. Bacterial and fungal contaminants easily grow in standard plant tissue culture media, quickly overgrowing plant cultures. Thus, it is important to discuss the major sources of contamination.

Comments on Work Area

Ideally, to reduce bacterial and fungal contamination significantly, plant tissue culture techniques should be performed in a laminar flow unit, and all surfaces, materials, and equipment should be disinfected or sterilized. A laminar flow unit filters the air flowing over the workspace, removing particles 0.3 micrometers or larger with greater than 99% efficiency. This greatly reduces the chance of airborne contamination. If a laminar flow unit is not available, these techniques may be performed with a good success rate on a table surface. The surface is wiped with 95% ethanol just before use. Since there is no sterile air flowing over the workplace, keep foot traffic down and work as quickly as possible to reduce airborne contamination.

Instrument Preparation and Use

Instruments used for dissecting and handling of plant material, such as scalpels and forceps, are placed in a hot oven (wrapped in heavy-duty aluminum foil) for three hours at 160° C for sterilization. During use, instruments are stored in 95% ethanol. It is not sufficient to use alcohol alone. Alcohol has little effect against bacterial spores and may even become contaminated with spores during use (Larson & Morton, 1991). Also, for this reason, a fresh solution should be used for each experiment.

All other nonsterile surfaces are wiped with 95% ethanol. This helps remove dust and does provide some disinfection. Due to the possibility of fire hazard, no flames are used.

The Culturer

For safety, and as an aid to aseptic technique, powderless gloves are worn. The gloves may be wiped with 95% ethanol. To reduce the chance of airborne contamination when working without a laminar flow unit, work as quickly as possible and do not work with your head directly over your workspace.

Disinfection of Plant Material

One major source of contaminants is the plant material itself. Bacterial and fungal contaminants cover the surfaces of plants. The inside tissues usually do not contain these organisms unless thay have been damaged by frost or injury. Viruses may still be present, but no steps are taken to remove them except for specialized procedures, such as meristem culture. Thus, disinfection is aimed at removal of surface contamination.

For this purpose, roots and underground plant parts are scrubbed under running tap water to remove residual soil, and then the outer layer is peeled using a vegetable scraper. Soft above-ground parts are not scrubbed but rinsed with tap water. Then these parts are exposed to a disinfectant solution, such as alcohol or bleach. For example, Brehm et al. (1996) found that 20% bleach provides a high percentage of uncontaminated explants.

In this unit, aseptic technique will be introduced. Exercise 7 will introduce preparation of instruments used for transferring cultures and will explore contamination during usage. In Exercise 8, two procedures for disinfection of plant material will be evaluated.

References

Brehm, M.S., Buguliskis, J.S., Hawkins, D.K., Lee, E.S., Sabapathi, D. & Smith, R.A. (1996). Determining the differences in efficacy of two disinfectants using *t* tests. *The American Biology Teacher, 58,* 111-113.

Larson, E.L. & Morton, H.E. (1991). Alcohols. In S.S. Block (Ed.), *Disinfection, Sterilization, and Preservation* (4th ed.), 191-203. Philadelphia, PA: Lea & Febiger.

7. Sterilization of Instruments

Objectives

1. To differentiate between **sterilization** and **disinfection**.

2. To understand how to reduce the chance of contamination while transferring plant tissue cultures.

Background

Plant tissue culture is the growing of isolated plant parts **aseptically** on appropriate media (Steward, 1983). In addition to the original explant material, major sources of contamination of plant tissue cultures are the instruments and other equipment used in their transfer. In microbiology, cultures are transferred using inoculating loops that are **sterilized** by holding them in the flame of a Bunsen burner until it is red hot. The instruments used for the transferring of plant tissue cultures, however, are not designed to be sterilized repeatedly in this manner (although some culturers still flame their instruments).

Another commonly used technique is to first **sterilize** (complete removal of all organisms including viruses and spores of bacteria) instruments, for example, by heating them for three hours at 160° C and then disinfecting them during use by immersing them in a disinfectant solution, such as 70% to 95% ethanol or isopropyl alcohol. Alcohols are disinfectants since they are not effective against bacterial spores (Larson & Morton, 1991).

A third practice is to start with nonsterile instruments and to immerse them in an alcohol solution prior to and during use. If alcohol is not very effective against spores, then it follows that there should be more contamination of cultures when alcohol is the only treatment used for instrument preparation. In addition, even with prior sterilization, instruments may become contaminated with each use.

In this exercise, the effectiveness of 95% ethanol in the disinfection of instruments contaminated with *Staphylococcus aureus*, a bacterium that does not produce spores, will be tested.

References

Larson, E.L. & Morton, H.E. (1991). Alcohols. In S.S. Block (Ed.), *Disinfection, Sterilization, and Preservation* (4th ed.), 191-203. Philadelphia, PA: Lea & Febiger.

Steward, F.C. (1983). Reflections on aseptic culture. In D.A. Evans, W.R. Sharp, P.V. Ammirato & Y. Yamada (Eds.), *Handbook of Plant Cell Culture, Volume I*, 1-10. New York: Macmillan Publishing Company.

Materials

(per student pair)

- 2 spatulas, sterile
- 1 20-mm x 150-mm culture tube containing 20 ml 95% ethanol
- 1 20-mm x 150-mm culture tube containing sterile distilled water
- 1 18- to 24-hour Brain Heart Infusion agar plate culture of *Staphylococcus aureus*
- 4 15-mm x 100-mm plates of sterile Brain Heart Infusion (BHI) agar, 20 ml per plate

Additional Materials

- incubator set at 35° C

Procedure

1. Use a marker to draw a line on the bottom of each of four sterile BHI agar plates, dividing them in half (see Figure 7.1).

2. Each student should remove one sterile spatula from its aluminum foil wrapper, touching only the handle.

3. One student should wipe the tip of his/her spatula

over one half of the first BHI plate, and the other student in the pair will wipe the tip of his/her spatula over the other half of the plate. This **before treatment** plate is the **negative** control to determine if bacteria are present prior to treatment.

4. Now, each student should wipe his/her spatula over the *S. aureus* growth and then immediately wipe the spatula onto the surface of a second sterile BHI plate. This will be the **positive** control. That is, this treatment will simulate contamination.

5. Rewipe the spatulas over the *S. aureus* growth.

6. Now, one student in the pair should dip his/her spatula into a tube of sterile distilled water for 10 seconds while the second student dips his/her spatula into a tube containing 95% ethanol for 10 seconds. Following this 10-second treatment, each of these spatulas is wiped over the surface of a third sterile BHI plate to determine if bacteria are present.

7. Rewipe each spatula over the *S. aureus* growth.

8. Now, one student should dip his/her spatula into a tube of sterile distilled water for 10 minutes while the second student dips his/her spatula into a tube containing 95% ethanol for 10 minutes. Following this 10-minute treatment, each of these spatulas are wiped over the surface of a fourth BHI plate.

9. Incubate the plates for 48 hours at 35° C and examine for growth.

10. Record results in the table provided and answer all questions on the report sheet provided.

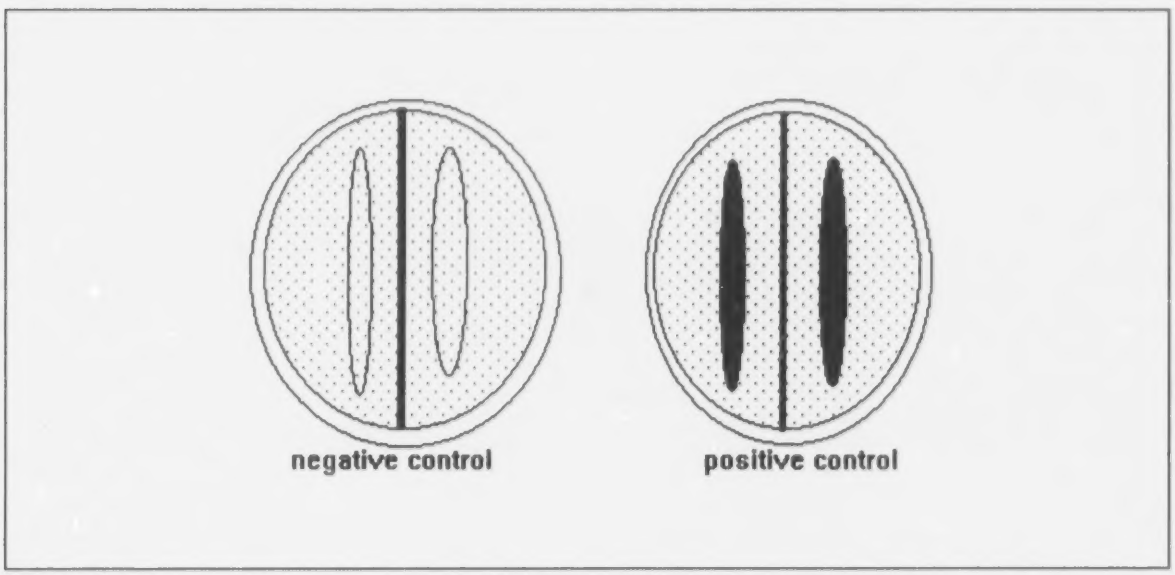

Figure 7.1. Appearance of uncontaminated (negative control) and contaminated (positive control) BHI plates after 48 hours of incubation.

Report 7
Sterilization of Instruments Name:

1. Record the presence or absence of growth for each treatment in the following table:

BHI plate	growth present	growth absent
Before plate, student 1		
Before plate, student 2		
After *S. aureus*, student 1		
After *S. aureus*, student 2		
After 10 seconds in distilled water		
After 10 seconds in 95% ethanol		
After 10 minutes in distilled water		
After 10 minutes in 95% ethanol		

2. What are the **specific questions** being asked?

3. Which treatment appears to be most effective, if any? Explain.

4. Would the results be the same if a spore-producing bacteria, such as *Bacillus subtilis,* were used instead of *S. aureus?* Explain.

5. Design an experiment using a one-way analysis of variance to determine the effectiveness of 95% ethanol for different time periods.

Optional

Design a study of your own using the investigation form provided in Appendix E. Following are some suggested questions:

1. Would 70% alcohol work better than 95%?
2. Would isopropyl alcohol work better than ethanol?

8. Disinfection of Plant Material

Objectives

1. To evaluate two procedures for disinfection of plant material.

2. To understand the effectiveness of the plant disinfection process.

Background

Bacterial and fungal contaminants on surfaces of plant material must be removed prior to their culture. A high percentage of uncontaminated explants can be obtained using 20% bleach for disinfection of sweet potato roots (Brehm et al., 1996). With some cultures, such as shoot tips and leaf tissue, an additional step frequently used is to rinse the explants with ethanol prior to bleach disinfection. The alcohol may act as a wetting agent, increasing the effectiveness of the bleach (Hu & Wang, 1983).

In this exercise, contamination of African violet leaf cultures disinfected with bleach alone will be compared to contamination of African violet leaf cultures treated with 95% ethanol prior to bleach disinfection.

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Materials

(per student pair)

- 2 scalpels, sterile
- 2 large forceps, sterile
- 2 300-ml beakers containing 200 ml 95% ethanol
- 1 African violet plant
- 6 300-ml flasks, each containing 250 ml sterile distilled water
- 2 300-ml beakers, covered with aluminum foil and sterilized
- 2 sterile petri plates (100 mm x 15 mm)
- 20% (v/v) bleach solution, about 500 ml
- 2 petri plates (100 mm x 15 mm), each containing 20 ml Murashige and Skoog salt base supplemented with 80 mg/L adenine sulfate, 2 mg/L IAA, 2 mg/L kinetin, 100 mg/L inositol, 0.4 mg/L thiamine HCl, 170 mg/L $NaH_2PO_4.H_2O$, 30 g/L sucrose, and 8 g/L agar.

Additional Materials

- growth chamber set at 25° C with a 16-hour photoperiod (approximately 2,000 lux)
- 95% ethanol, about 100 ml
- parafilm

Procedure

African Violet Leaf Disinfection

1. Place one set of sterile instruments in each of the 300-ml beakers containing 95% ethanol.

2. Remove the younger leaves from the center portion of an African violet *(Saintpaulia ionantha)* plant purchased from a grocery store. Each student pair will use two leaves.

3. Disinfect one leaf in an aluminum foil-covered beaker filled with 20% (v/v) bleach solution. Swirl the leaves in this solution every few minutes for 10 minutes. Then rinse three times using sterile distilled water.

4. The second student in the pair will disinfect his/her leaf in an aluminum foil-covered beaker first by swirling it for one minute with enough 95% etha-

nol to cover it, followed by swirling it for 10 minutes in 20% (v/v) bleach solution. Rinse three times using sterile distilled water.

Explant Preparation

1. Using forceps, each student will place his/her leaf, with the lower surface facing up, in a sterile petri dish (100 mm x 15 mm).
2. Holding the leaf steady with forceps, use the scalpel to cut and remove the outer edges of the leaf blade, leaving the center portion of the blade, including the midvein (Figure 8.1). Cut this center portion transversely into explants 5-mm wide.
3. Transfer five explants to an agar plate, keeping them well separated. Press one end of each section one-fourth into the medium.
4. If more replications are desired, other student pairs should follow the same procedure.
5. Wrap plates with parafilm and place in a growth chamber at 25° C with a 16-hour photoperiod (approximately 2,000 lux) for four weeks.
6. Record contaminations in the table provided and answer the questions on the report sheet.

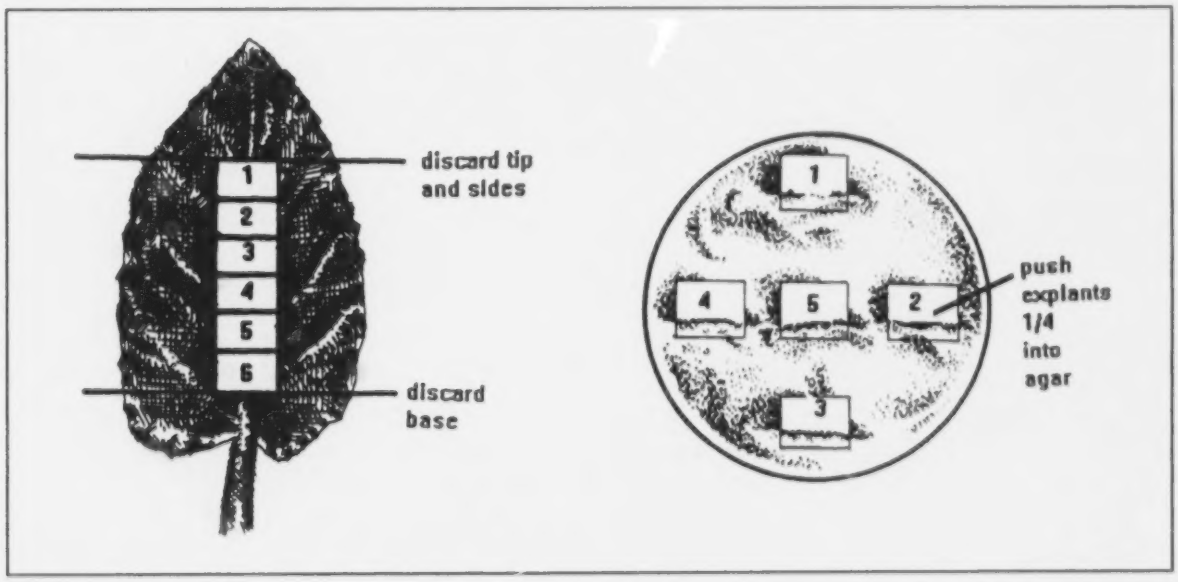

Figure 8.1. Preparation and culture of African violet explants.

Report 8
Disinfection of Plant Material

Name:

1. Record the number of uncontaminated explants for each treatment in the following table. There is enough room for 9 student pairs.

Student Pair	Bleach Disinfected Explants	Alcohol Plus Bleach Disinfected Explants
1		
2		
3		
4		
5		
6		
7		
8		
9		
Mean		

2. Before performing statistical analysis, examine the data. Which treatment appears to be most effective, if any? Explain.

3. For statistical analysis since we are comparing two independent samples:

   a. Which statistical procedure is appropriate for analysis of the data?

   b. What is the **specific question**?

c. What is the **null hypothesis**?

d. Is this **one-tailed** or **two-tailed**? Explain.

e. State the **alternative hypothesis**.

4. Perform the appropriate statistical analysis and record below.

5. Is it necessary to pretreat African violet leaves with alcohol or is bleach disinfection sufficient?

a. Is there a statistical significance?

b. Is there a practical significance?

Optional

Design a study of your own, using the investigation form provided in Appendix E. Following are some suggested questions.

1. Would isopropyl alcohol work better than ethanol?
2. Would adding a drop of detergent to the bleach work better than using alcohol?
3. What would be the effect of increasing the number of replications to 20? To 30?

UNIT III – CULTURE METHODS

Plant tissue culture methods include the initiation and establishment of callus cultures, cell suspension cultures, the culture of single cells, shoot tip and meristem cultures, root tip cultures, anther cultures, and embryo cultures. Once cultures are established, they may be used for basic propagation techniques, such as organogenesis, embryogenesis, and shoot tip culturing, or for specialized techniques, such as production of secondary metabolites, bioassays, protoplast isolation and fusion, genetic transformation, or production of new plant varieties. In this unit, the initiation and maintenance of callus, suspension, single cell, and shoot tip cultures will be explored.

Callus Culture

Callus may be established from most excised plant parts. Callus is a mass of cells that grows from the excised plant part consisting of irregularly differentiated, vacuolated cells interspersed with smaller meristematic cells (Hussey, 1986). Once established, the callus tissue may be subdivided every three to four weeks and cultured indefinitely as callus on the same medium. Alternatively, the callus may be transferred to media with different concentrations of hormones for embryogenesis (Ammirato, 1983) or organogenesis (Flick, 1983) or used for establishment of cell suspension cultures. One characteristic of callus culture is that, as the cells proliferate, genetic changes, such as polyploidy, aneuploidy, and chromosome structure changes can occur (Reisch, 1983; Escandon et al., 1985). Although there is more variation seen in these callus cells than in the plants regenerated from them, new varieties of plants with useful characteristics have been obtained by this technique (Reisch, 1983).

Cell Suspension Culture

A suspension of individual cells and small cell clusters can be obtained by placing callus into a liquid medium and incubating on a shaker. As new cells are formed, the shaking causes some of them to separate from the callus. These cultured cells are not genetically stable (Reisch, 1983). Advantage can be taken of this by plating out these suspension cultures and screening for variant cell lines (Flick, 1983). Other applications of suspension cultures include production of secondary metabolites (Constabel & Vasil, 1988; Fowler, 1986), studies of plant organelles (Fowke, 1987), and embryogenesis (Ammirato, 1983).

Culture of Single Cells

Individual cells and cell clusters from suspension cultures can be cultured on agar solidified media. Cell cultures are useful for studies such as isolation of mutants (Flick, 1983), stress tolerance (Nabors et al., 1975), and somoclonal variation (Larkin & Snowcroft, 1981; Creissen & Karp, 1985).

Shoot Tip Cultures

Shoot tip cultures, about 1 mm or more in length and consisting of the apical meristem, several leaf primordia, and subapical stem tissue, are used for rapid asexual propagation of many economically important plants (Hu & Wang, 1983; Rice, 1992). In addition, they have been studied as a means for production of metabolites in culture (Heble, 1985). A more difficult technique is the culturing of only the shoot apical meristem; however, this technique is useful for elimination of viruses and other pathogens. Shoot tips derived from apical meristem cultures then may be used for germplasm preservation (Kartha, 1987) and production of disease-free stocks (Wenzel, 1992).

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9. Sweet Potato Root Callus Culture

Objectives

1. To gain practice establishing and maintaining a callus culture from the sweet potato root.

2. To gain experience in determining growth curves using fresh and dry weight measurements.

Background

To establish a callus culture, the plant material to be used first must be disinfected. Then, small pieces of the plant, known as *explants*, are placed on a medium containing concentrations of hormones designed to stimulate growth. Dedifferentiated masses of cells, known as *callus*, form from cells of the explant. About every four to eight weeks, the explant with callus is divided and subcultured to fresh medium. Eventually, enough callus material is produced that none of the original explant has to be subcultured. The callus then may be subcultured as long as needed.

The frequency of subculturing depends upon the growth rate of the callus. Growth rates may be established by plotting increase in weight or some other measurement against time to produce a growth curve. Growth curves may resemble an idealized S-shaped growth curve, with lag, exponential, linear, and stationary phases (Figure 9.1). These curves then can be used to determine the effects of an experimental treatment, such as combinations of growth regulators, on the growth of the callus. The goal may be to maximize growth or may simply be to determine the optimum culture period for some other procedure, such as protoplast isolation or the harvesting of secondary metabolites.

In this exercise, growth curves for sweet potato callus cultures will be determined using fresh weight and dry weight measurements.

Materials

(per student pair)

- 1 scalpel, sterile
- 1 large forceps, sterile
- 1 utility tongs, sterile
- 1 cork borer (5 or 6 mm in diameter) with punch, sterile
- 1 300 ml beaker containing 250 ml 95% ethanol
- 1 sweet potato root
- 3 300-ml flasks, each containing 250 ml sterile distilled water
- 1 600-ml beaker, covered with aluminum foil and sterilized
- 10 sterile petri plates (100 mm x 15 mm)
- bleach solution, 20% (v/v), 500 ml
- 50 culture tubes (150 mm x 20 mm), each containing 8 ml Murashige and Skoog salt base supplemented with 1 mg/L 2,4-D, 100 mg/L inositol, 0.4 mg/L thiamine HCl, 0.5 mg/L pyridoxine HCl, 0.5 mg/L nicotinic acid, 2 mg/L glycine, 1 g/L casein hydrolysate, 30 g/L sucrose, and 8 g/L agar.

Additional Materials

- vegetable scraper
- kitchen knife
- parafilm
- incubator set at 25° C
- ruler with mm markings
- analytical balance
- aluminum weighing pans
- oven set at 60° C

Procedure

Sweet Potato Root Disinfection

1. Sweet potatoes *(Ipomoea batatas)*, purchased from a grocery store, are scrubbed thoroughly under running tap water to remove residual soil.

2. Remove the skin using a vegetable scaper and then cut the potato transversely into slices about 15 mm thick. Each student pair will need six slices.

3. Disinfect the slices in an aluminum foil-covered

beaker filled with 20% (v/v) bleach solution. Swirl the slices in this solution every few minutes for 10 minutes. Then rinse three times using sterile distilled water.

Explant Preparation

1. Place sterile instruments in the beaker containing the 95% ethanol.

2. Using the utility tongs, place each slice in a separate sterile petri dish (100 mm x 15 mm).

3. Hold the slice steady with the tongs. Make borings with the cork borer parallel to the vertical axis of the tissue slice. Avoid including any of the side surface from the slice since this was damaged by the disinfectant.

4. Push the cork borer completely through the slice. While holding the slice steady with the tongs, remove the cork borer. The boring will remain inside the borer.

5. Plunge the boring into a second sterile petri dish using the cork borer punch. Repeat this process to obtain 10 borings from each slice, for a total of 60 borings.

6. Use a scalpel to remove about 2 mm of tissue from the end of each of the borings. Cylinders 10 mm in length should remain.

Culturing

1. Use forceps to transfer one explant to each tube of Murashige and Skoog medium until a total of 50 explants have been transferred. The remaining 10 explants will be used for "baseline" fresh and dry weight data and will not be cultured.

2. Incubate the tubes at 25° C. No light is necessary. Callus will be visible the first week of culture (Figure 9.2).

3. Starting with the 10 noncultured explants, the mean fresh weights of 10 explants must be determined to the nearest 0.1 mg each week for six weeks.

4. After fresh weight is determined, the same explants are used for dry weight determination by placing them in an oven at 60° C for 48 hours and then reweighing.

5. Record observations each week on the report sheet provided and answer the questions.

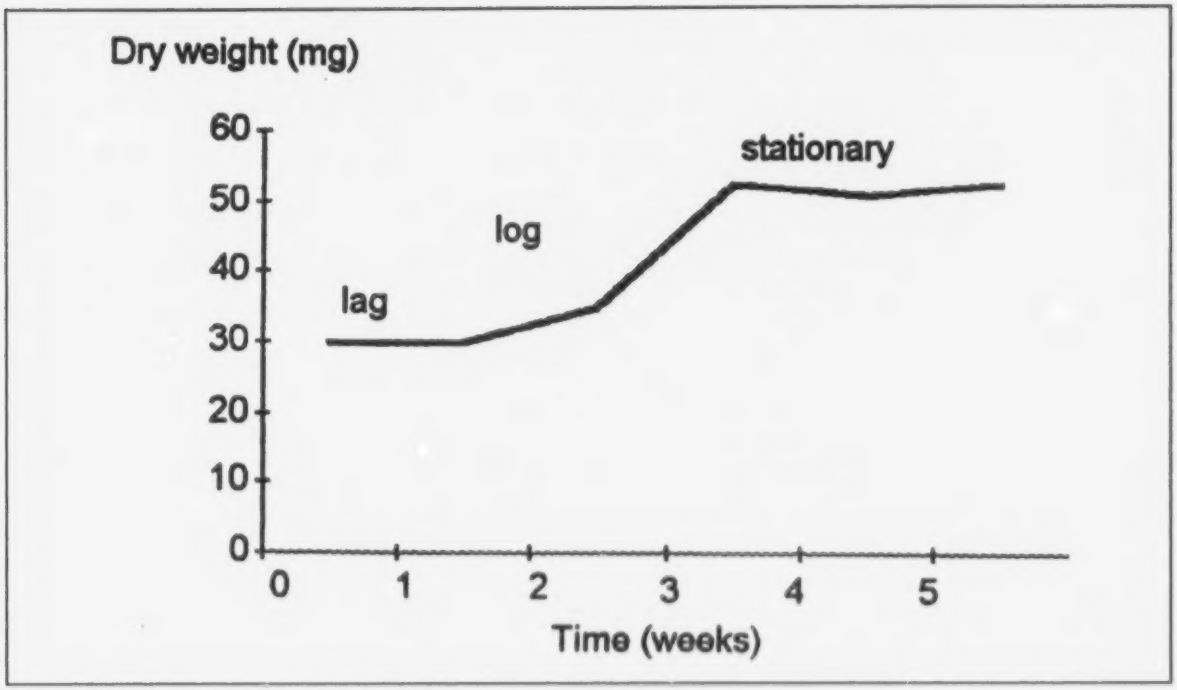

Figure 9.1. Growth curve of callus showing lag, log, and stationary phases of growth.

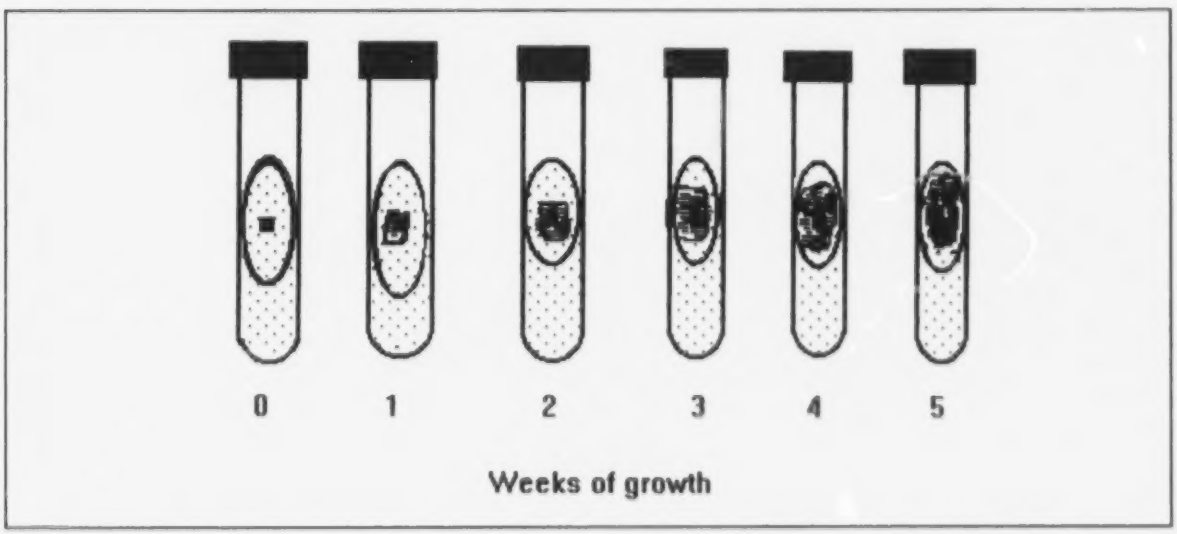

Figure 9.2. Change in sweet potato explant appearance. From left to right, at time of preparation and after one, two, three, four, and five weeks of growth.

Report 9
Sweet Potato Root Callus Culture Name:

1. On a weekly basis, record the fresh and dry weights of 10 explants in the following table:

	Fresh Weight	Dry Weight
Week 0		
Week 1		
Week 2		
Week 3		
Week 4		
Week 5		

2. Plot your data for both fresh weight and dry weight from the above table in the graph below:

3. Compare your growth curves to the sample growth curve in Figure 9.1. Are the growth curves for both fresh and dry weights similar to the sample growth curve? Describe any differences.

Optional

Design a study of your own using the investigation form provided in Appendix E. Following are some suggested questions:

1. What would be the effect of using explants 5-mm long on the shape of the growth curves?
2. What would be the effect on these growth curves if you removed the 1 g/L casein hydrolysate?

10. Carrot Root Callus Culture

Objective

To gain practice establishing and maintaining a callus culture from carrot root.

Background

The techniques used for establishing and maintaining carrot taproot cultures are similar to those used for sweet potato root cultures (Exercise 9). Callus initiation and development, however, do not occur at the same rate for all plant species, for different plant parts, or even for different size explants of the same species. In this exercise, the development of callus from carrot taproot explants of three different sizes will be compared.

Materials

(per student pair)

- 1 scalpel, sterile
- 1 large forceps, sterile
- 1 utility tongs, sterile
- 1 cork borer (5 or 6 mm in diameter) with punch, sterile
- 1 300-ml beaker containing 250 ml 95% ethanol
- 1 carrot taproot
- 3 300-ml flasks, each containing 250 ml sterile distilled water
- 1 600-ml beaker, covered with aluminum foil and sterilized
- 6 sterile petri plates (100 mm x 15 mm)
- bleach solution, 20% (v/v), 500 ml
- 3 petri plates (100 mm x 15 mm), each containing 20 ml Murashige and Skoog salt base supplemented with 1 mg/L 2,4-D, 100 mg/L inositol, 0.4 mg/L thiamine HCl, 0.5 mg/L pyridoxine HCl, 0.5 mg/L nicotinic acid, 2 mg/L glycine, 1 g/L casein hydrolysate, 30 g/L sucrose, and 8 g/L agar.

Additional Materials

- vegetable scraper
- kitchen knife
- parafilm
- vegetable brush
- incubator set at 25° C
- ruler with mm markings

Procedure

Carrot Taproot Disinfection

1. Carrots *(Daucus carota)*, purchased from a grocery store, are scrubbed thoroughly under running tap water to remove residual soil.

2. Remove the skin using a vegetable scaper and then cut the carrot transversely into slices about 15 mm thick. Each student pair will need three (3) slices.

3. Disinfect the slices in an aluminum foil covered beaker filled with 20% (v/v) bleach solution. Swirl the slices in this solution every few minutes for 10 minutes. Then rinse three times using sterile distilled water.

Explant Preparation

1. Place sterile instruments in the beaker containing the 95% ethanol.

2. Using the utility tongs, place each slice in a separate sterile petri dish (100 mm x 15 mm).

3. Hold one slice steady with the tongs. Make borings with the cork borer parallel to the vertical axis of the tissue slice, being sure to include the cambium (Figure 10.1).

4. Push the cork borer completely through the slice. While holding the slice steady with the tongs, remove the cork borer. The boring will remain inside the borer.

5. Plunge the boring into a second sterile petri dish using the cork borer punch. Repeat this process to obtain five borings from the slice.

6. Use a scalpel to remove about 2 mm of tissue from the end of each of the borings. A cylinder 10 mm in length should remain.

7. Also prepare five 5-mm cylinders from the second slice and five 2.5-mm cylinders from the third slice.

Culturing

1. Use forceps to transfer five explants to each plate (100 mm x 15 mm) of Murashige and Skoog medium. Arrange the explants as in Figure 2.1, Exercise 2, so that they rest flat on the agar surface and do not touch.

2. Wrap the dishes in parafilm and incubate the plates at 25° C. No light is necessary.

3. For four weeks, examine the plates for signs of callus development. Record observations on the report sheet and answer the questions.

Subculturing

1. Following four weeks of observations, transfer explants showing no contamination but an abundance of callus into a sterile petri dish.

2. Using a sterile scalpel, divide the callus into pieces about 5 mm x 5 mm. Transfer five pieces to each plate, keeping them well separated.

3. Incubate at 25° C. No light is necessary.

4. Examine cultures weekly for contamination and discard infected cultures. Every three to four weeks, divide and transfer uncontaminated callus to fresh medium.

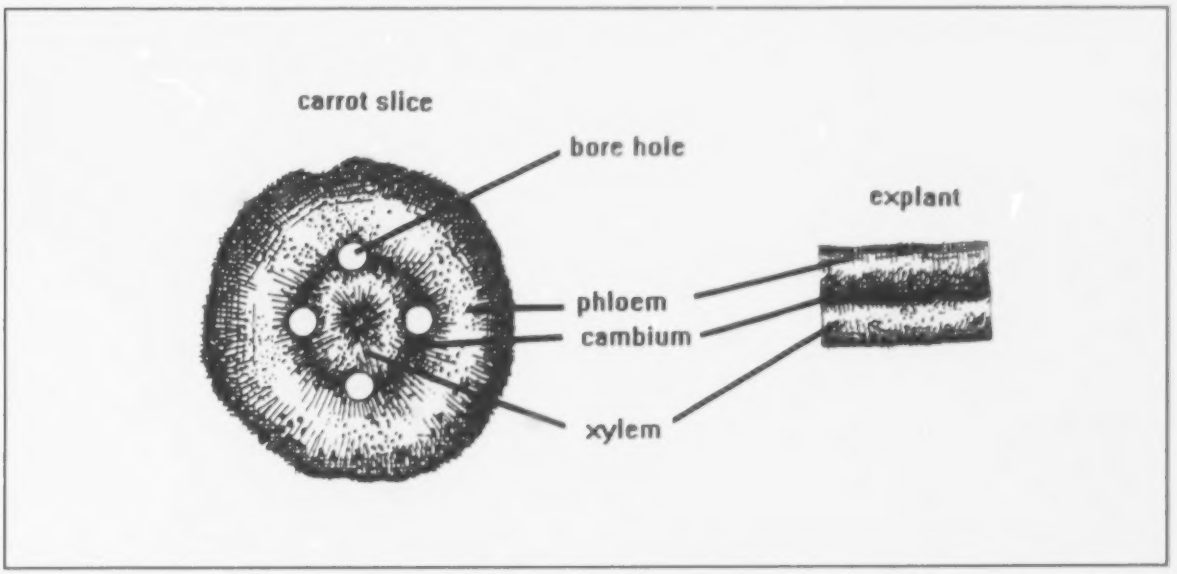

Figure 10.1. Carrot taproot explants.

Report 10
Carrot Root Callus Culture Name:

1. On a weekly basis, record the percentage of explants showing callus development for each explant size in the following table:

Explant Size	Percent Callus Week 1	Percent Callus Week 2	Percent Callus Week 3	Percent Callus Week 4
10 mm				
5 mm				
2.5 mm				

2. On a weekly basis, record the percentage of explants showing contamination for each explant size in the following table:

Explant Size	Percent Contamination Week 1	Percent Contamination Week 2	Percent Contamination Week 3	Percent Contamination Week 4
10 mm				
5 mm				
2.5 mm				

3. Following four weeks incubation, which explant size appears to have produced the most callus? The most contamination? Explain.

4. In this exercise, we compared callus and percent contamination. Now, design an experiment for comparing change in fresh weight following four weeks of incubation for each explant size. Which statistical procedure would be appropriate now? Explain.

Optional

Design a study of your own using the investigation form provided in Appendix E. Following are some suggested questions:

1. Would callus development occur at a different rate if roots from carrot seedlings were used?
2. Does use of callus from different sources (taproot, seedling, root, hypocotyl) influence results for embryogenesis? For organogenesis? For secondary products?

11. Tobacco Leaf Cultures

Objective

To gain practice establishing and maintaining cultures from tobacco leaves.

Background

Leaf explants, including cotyledonary tissue, may be used for **callus** production to study **organogenesis**, **secondary product formation**, or for studies of **transformation** using *Agrobacterium tumefaciens* (Prosen & Simpson, 1987; Fillatti et al., 1987; Tabata & Hiraoka, 1976; Burtin et al., 1990: Hinchee et al., 1988; Flick et al., 1983). Tobacco is useful as a model system for many of these studies since results are specific and reproducible. In this exercise, callus and shoot cultures from tobacco leaves will be initiated and maintained.

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Materials

(per student pair)

- 1 scalpel, sterile
- 1 large forceps, sterile
- 1 cork borer (5 or 6 mm in diameter) with punch, sterile
- 1 300-ml beaker containing 250 ml 95% ethanol
- 1 tobacco plant *(Nicotiana tabacum* cv. Wisconsin No. 38)
- 3 300-ml flasks, each containing 250 ml sterile distilled water
- 1 600-ml beaker, covered with aluminum foil and sterilized
- 4 sterile petri plates (100 mm x 15 mm)
- 500 ml 20% (v/v) bleach solution containing one drop of liquid detergent
- 2 petri plates (100 mm x 15 mm), each containing 20 ml Murashige and Skoog salt base supplemented with 1 mg/L 2,4-D, 100 mg/L inositol, 0.4 mg/L thiamine HCl, 0.5 mg/L pyridoxine HCl, 0.5 mg/L nicotinic acid, 2 mg/L glycine, 1 g/L casein hydrolysate, 30 g/L sucrose, and 8 g/L agar.
- 2 petri plates (100 mm x 15 mm), each containing 20 ml Murashige and Skoog salt base supplemented as above but using 1.1 mg/L BA instead of 2,4-D.

Additional Materials

- parafilm
- growth chamber set at 25° C and a 16-hour photoperiod (approximately 2,000 lux)

Procedure

Tobacco Leaf Disinfection

1. Remove the youngest leaves from a tobacco plant (*Nicotiana tabacum* cv. Wisconsin No. 38) and dis-

infect in an aluminum foil covered beaker filled with 20% (v/v) bleach solution. Each student will need two leaves.

2. Swirl the leaves in this solution every few minutes for 10 minutes. Then rinse three times using sterile distilled water.

Explant Preparation

1. Place sterile instruments in the beaker containing 95% ethanol.

2. Using the forceps, place each leaf in a separate sterile petri dish.

3. Hold the leaf steady with the forceps. Make borings with the cork borer. The boring will remain inside the borer.

4. Plunge the boring into a second sterile petri dish using the cork borer punch.

5. Repeat this process to obtain 10 borings from each leaf. A total of 20 borings will be needed.

Culturing

1. Use forceps to transfer five explants to each plate of Murashige and Skoog medium. This will give a total of10 explants on media containing 2,4-D and 10 explants on media containing BA.

2. Arrange the explants as in Figure 2.1, Exercise 2, so that they rest flat on the agar surface and do not touch.

3. Wrap plates with parafilm and incubate plates at 25° C with a 16-hour photoperiod (approximately 2,000 lux).

4. For four weeks, examine the plates weekly for signs of callus or shoot development. Record observations on the report sheet and answer the questions.

Subculturing of Callus

1. Following four weeks of observations, transfer explants showing no contamination but an abundance of callus into a sterile petri dish (100 mm x 15 mm).

2. Using a sterile scalpel, divide the callus into pieces about 5 mm x 5 mm.

3. Transfer five pieces to each plate of fresh medium, keeping them well separated and incubate the plates as above.

5. Examine the cultures weekly for contamination and discard infected cultures.

6. Every three to four weeks continue to divide and transfer uncontaminated callus to fresh media.

Subculturing of Shoots

1. Shoots may be separated and transferred to fresh medium containing BA to maintain as shoot cultures.

2. For plantlet production, shoots one inch or more in length are transferred to Jiffy pellets. Enclose pellets in plastic bags to maintain humidity and incubate at 25° C in low light. Once roots emerge from the pellet, transfer to pots and gradually expose to normal light and humidity.

Report 11
Tobacco Leaf Culture

Name:

1. On a weekly basis, record the percentage of explants showing callus or shoot development for each medium in the following table:

Medium	Percent Callus or Shoots Week 1	Percent Callus or Shoots Week 2	Percent Callus or Shoots Week 3	Percent Callus or Shoots Week 4
Murashige and Skoog with 2,4-D				
Murashige and Skoog with BA				

2. Following four weeks incubation, which medium appears to have produced the most callus? The most shoots? Explain.

3. How does tobacco leaf callus development compare to sweet potato root and carrot taproot callus development?

Optional

Design a study of your own using the investigation form provided in Appendix E. Following are some suggested questions:

1. Would callus development occur at a different rate if cotyledons from tobacco seedlings were used?
2. Would use of callus from tobacco stem pith instead of tobacco leaves affect organogenesis?

12. Sweet Potato Suspension Cultures

Objectives

1. To gain practice establishing and maintaining suspension cultures from sweet potato callus.

2. To gain experience determining growth curves for suspension cultures.

Background

Cell suspension cultures are easily initiated from callus cultures by transferring the callus into a liquid medium of the same composition as the solid medium, except that agar is omitted. The suspension is then subjected to continual shaking or stirring. Cells and cell clusters separate from the callus with the constant movement. The suspensions may be subcultured every two to three weeks by transferring a portion of the suspension to fresh medium. Growth curves (Figure 12.1) are generally established to determine optimum conditions for subculturing. For example, an important variable affecting the growth pattern is initial inoculation density. If inoculation density is too low, growth will be slow or may not occur at all. Growth curves for suspension cultures may be determined by measuring changes in fresh and dry weights, settled cell and packed cell volumes, cell number, mitotic index, or protein content (Bellincampi et al., 1985; Henshaw et al., 1966; Yeoman et al., 1965; Hahlbrock & Kuhlen, 1972; Manasse, 1972; Schmauder et al., 1985). The advantage of packed cell volume and settled cell volume is that they are nondestructive; that is, the culture does not have to be sacrificed.

In this exercise, determination of growth curves of sweet potato suspension cultures will be compared using settled-cell and packed-cell volumes as the means of measurement.

References

Bellincampi, D., Baduri, N. & Morpurgo, G. (1985). High-plating efficiency with plant cell cultures. *Plant Cell Reports, 4,* 155-157.

Hahlbrock, K. & Kuhlen, E. (1972). Relationship between growth of parsley and soybean cells in suspension cultures and changes in conductivity of the culture medium. *Planta, 108,* 271-278.

Henshaw, G.G., Jha, K.K., Mehta, A.R., Shakeshaft, D.J. & Street, H.E. (1966). Studies on the growth in culture of plant cells. I. Growth patterns in batch propagated suspension cultures. *Journal of Experimental Botany, 17,* 362-377.

Manasse, R.J. (1972). A new culture flask for plant tissue suspension cultures. *Experientia, 28,* 723-724.

Schmauder, H.P., Groger, D., Koblitz, H. & Koblitz, D. (1985). Shikimate pathway activity in shake and fermenter cultures of *Cinchona succirubra. Plant Cell Reports, 4,* 233-236.

Yeoman, M.M., Dyer, A.F. & Robertson, A.I. (1965). Growth and differentiation of plant tissue cultures. *Annals of Botany, 29,* 265-276.

Materials

(per student pair)

- 1 spatula, sterile
- 1 300-ml beaker containing 250 ml 95% ethanol
- 1 callus culture of sweet potato *(Ipomoea batatas)* growing in Murashige and Skoog medium (as described in Exercises 9 and 10) and four weeks since the last subculture.
- 3 250-ml Erlenmeyer flasks each containing 35 ml of Murashige and Skoog salt base supplemented with 1 mg/L 2,4-D, 100 mg/L inositol, 0.4 mg/L thiamine HCl, 0.5 mg/L pyridoxine HCl, 0.5 mg/L nicotinic acid, 2 mg/L glycine, 1 g/L casein hydrolysate, and 30 g/L sucrose.

Additional Materials

- sterile 50-ml centrifuge tubes
- sterile 10-ml pipets, wide tips

- bench top centrifuge
- rotary shaker

Procedure

Initiation of Suspension Cultures

1. Transfer about two grams of an established sweet potato callus culture, as described in Exercises 9 and 10 and obtained four weeks since the last subculture, to one of the flasks containing 35 ml of Murashige and Skoog medium.
2. Incubate for three to four weeks on a rotary shaker at 150 rpm at 25° C. No light is necessary for incubation of the flask.

Inoculation of Flasks

1. Thoroughly mix the established suspension culture.
2. Using a wide-bore pipet, transfer 15 ml from this culture to each remaining flask of fresh media.
3. Label one flask to be used for settled cell volume and one flask for packed cell volume.

Settled-Cell Volume

1. Transfer the contents of the settled-cell volume flask to a sterile 50-ml centrifuge tube.
2. Allow the cells to settle for 30 minutes. Determine the settled-cell volume (Figure 12.2).
3. Record the volume as time zero settled-cell volume in the table provided.
4. Transfer the suspension back to its original 250-ml Erlenmeyer flask.
5. Incubate on a rotary shaker at 150 rpm at 25° C. No light is necessary.
6. Every three or four days, determine and record settled-cell volume.

Packed-Cell Volume

1. Transfer the contents of the packed-cell volume flask to a sterile conical 50-ml centrifuge tube.
2. Centrifuge at 1,000 g for five minutes.
3. Record the volume as time zero packed-cell volume in the table provided.
4. Transfer the suspension back to its original 250-ml Erlenmeyer flask.
5. Incubate on a rotary shaker at 150 rpm at 25° C. No light is necessary.
6. Every three or four days, determine and record packed-cell volume.
7. Answer all questions on the report sheet that follows this activity.

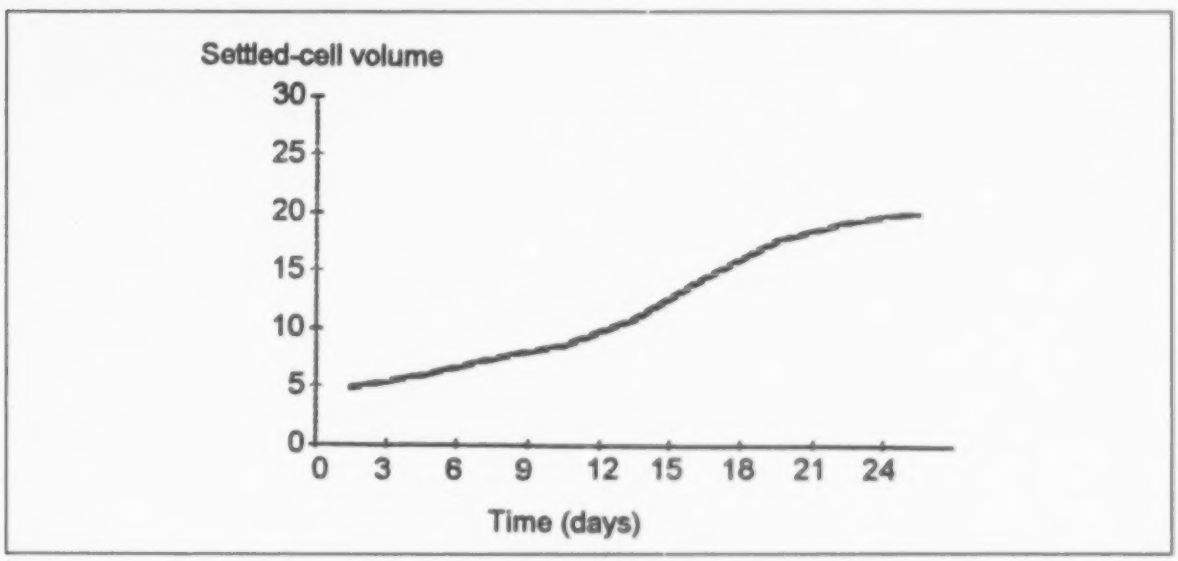


Figure 12.1. Settled-cell volume growth curve for a sweet potato suspension culture.

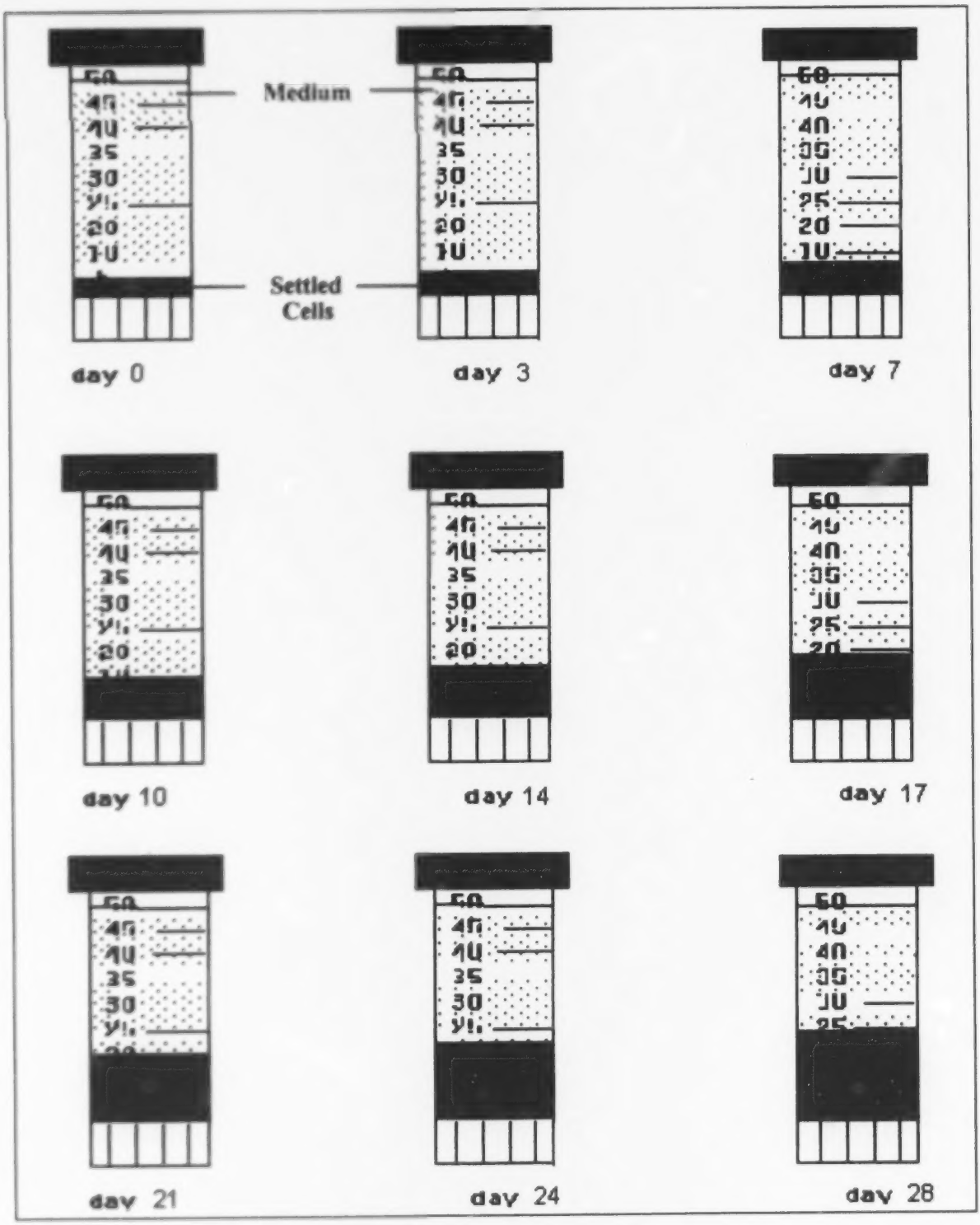


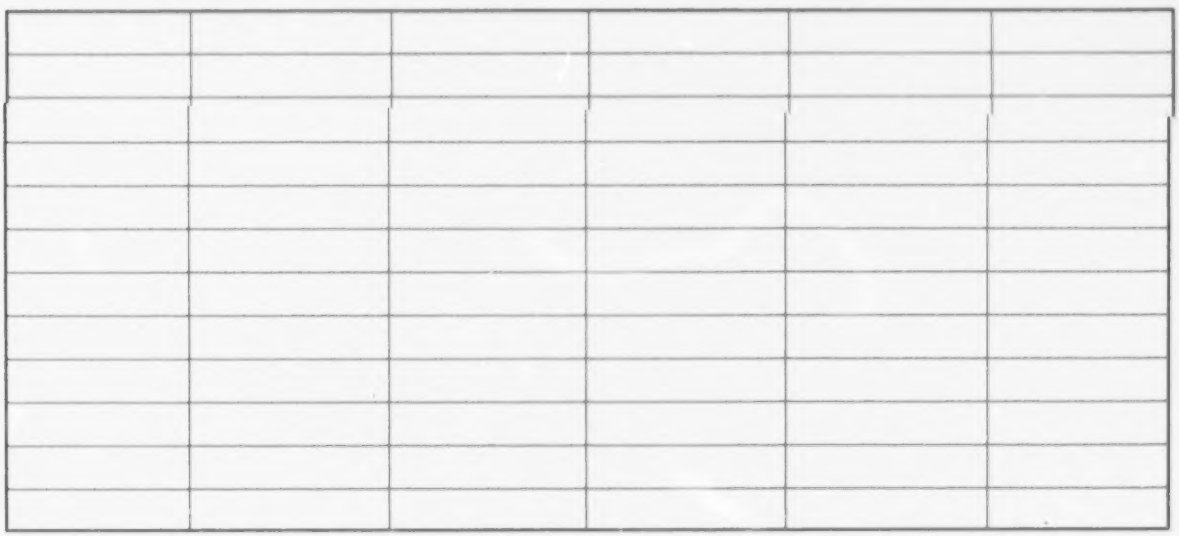
Figure 12.2. Settled-cell volumes for sweet potato suspension cultures at days 0, 3, 7, 10, 14, 17, 21, 24, and 28.

Report 12
Sweet Potato Suspension Cultures Name:

1. Record the settled-cell and packed-cell volumes in the following table:

	Settled-Cell Volume	Packed-Cell Volume
Day 0		
Day 3		
Day 7		
Day 10		
Day 14		
Day 17		
Day 21		
Day 24		
Day 28		

2. Plot your data for settled-cell volume and packed-cell volume from the table on page 82 on the graph below.



3. Compare growth curves for the two parameters. Describe any differences.

Optional

Design a study of your own using the investigation form provided in Appendix E. Following are some suggested questions:

1. What would be the effect of using an inoculation size of 5 ml on the shape of the growth curves?
2. What would be the effect on these growth curves if you removed one of the supplements, such as the 2,4-D?

13. Sweet Potato Cell Cultures

Objectives

1. To gain practice establishing cell cultures from sweet potato callus.

2. To gain experience in using plating efficiency as a means for determining growth in cell cultures.

Background

Cell suspension cultures may be plated out and used to establish single cell cultures. One problem associated with this technique is that a large inoculation size, about 1,000 to 10,000 cells/ml (Kao & Michayluk, 1975; Bellincampi et al., 1985) may be required to obtain colonies. On the other hand, lower densities would facilitate subsequent isolation and characterization of cell lines (Bellincampi et al., 1985).

Bellincampi et al. (1985) used a two-stage plating technique to overcome this problem. That is, cells were inoculated at high density (1,000 cell units/ml) in a 0.2% agarized medium and allowed to form microcolonies first; then a limited number of these colonies were separated and plated on solid media for production of colonies.

In another set of experiments, Kao and Michayluk (1975) found that an inoculation density of about 5,000 cells/ml was required for *Vicia hajastana* cells grown on a modified Gamborg's medium supplemented with sucrose, glucose, vitamins, and 2,4-D. They were able to decrease the required population density for these cells to 1 to 2 cells/ml by adding a number of metabolic intermediates, coconut milk, and casamino acids to this medium. They concluded that the simplest way to grow cells at very low density is to supplement the medium with coconut water and casein hydrolysate.

In this exercise, the influence of casein hydrolysate on plating efficiency of sweet potato cells will be examined. This will be tested using Murashige and Skoog medium, with or without casein hydrolysate. Plating efficiency is the number of cells or clusters that grow into visible colonies divided by the number of such units plated (Bellincampi et al., 1985).

References

Bellincampi, D., Baduri, N. & Morpurgo, G. (1985). High plating efficiency with plant cell cultures. *Plant Cell Reports, 4,* 155-157.

Kao, K.N. & Michayluk, M.R. (1975). Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. *Planta, 126,* 105-110.

Materials

(per student pair)

- 1 sweet potato *(Ipomoea batatas)* suspension culture, two weeks since the last subculture, growing in a 250-ml Erlenmeyer flask containing 50 ml Murashige and Skoog salt base, supplemented with 1 mg/L 2,4-D, 100 mg/L inositol, 0.4 mg/L thiamine HCl, 0.5 mg/L pyridoxine HCl, 0.5 mg/L nicotinic acid, 2 mg/L glycine, and 30 g/L sucrose.
- 4 petri plates (100 mm x 15 mm), each containing 20 ml Murashige and Skoog salt base supplemented as above with the addition of 8 g/L agar.
- 4 petri plates (100 mm x 15 mm), each containing 20 ml Murashige and Skoog salt base supplemented as above with the addition of 1 g/L casein hydrolysate and 8 g/L agar.

Additional Materials

- sterile 50-ml centrifuge tubes
- sterile distilled water
- parafilm
- Eosinophil counting chamber
- 1-ml pipets
- incubator set at 25° C
- microscope with low and high power objectives

Procedure

Colony Counts

1. Transfer the suspension culture to a sterile 50-ml centrifuge tube and allow cells to settle for 30 minutes. Determine the settled-cell volume. Use this as a guide to set up one tube with a settled-cell volume of 5 ml.

2. Pour off spent medium and resuspend the cells using distilled water to the 50-ml mark. Shake well and pour 5 ml from this tube into a new sterile 50-ml centrifuge tube. Resuspend these cells with distilled water to the 50-ml mark. This is a 1/10 dilution.

3. Shake the second tube and pour 5 ml into a third 50-ml centrifuge tube. Add distilled water up to 50 ml for a 1/100 dilution.

4. Repeat this process again for a 1/1,000 dilution.

5. Shake all tubes well. Dispense 5 ml of each dilution on to the surface of media with and without casein hydrolysate.

6. Swirl each plate to evenly spread the cells. Incubate at 25° C for several days to allow surface water to evaporate and then seal with parafilm for the remainder of incubation. No light is necessary.

7. Following four weeks of incubation, count colonies larger than 1 mm in each plate (see Figure 13.1). Plates with more than 200 colonies are reported as being too numerous to count.

8. Record results in the table provided and answer all questions.

Detemination of Cell Number

1. The Speirs-Levy Eosinophil counting chamber has four chambers, each consisting of ten 1 x 1 mm squares subdivided into 16 smaller squares (Figure 13.2). The depth of the chambers is 0.2 mm, which allows the large plant cells to slide more easily under the cover slip during loading.

2. Mix the undiluted 5-ml SCV tube again to resuspend the cells.

3. Place the cover glass over the counting chambers and use a 1-ml pipet to transfer a small amount of the suspension to the slide by touching the edge of the cover glass with the pipet tip and allowing the suspension to flow by capillary action. Alternatively, place a drop of the mixture over each counting chamber and then place the cover slip over the drops, avoiding air bubbles.

4. Let the counting cell stand for five minutes to allow the cells to settle.

5. Start with chamber 1. Count all the cells within each of the 10 one-square-millimeter areas and those touching the ruled lines on the left and upper part of the chambers. If cells are too numerous or too few (<20/chamber) to count, use another dilution to provide a desired range of cells.

6. Use the same procedure to court the cells in the other three chambers and determine the average count per chamber (10 square millimeters).

7. The formula for calculating the number of cells per ml is:

$$\frac{\text{Average number of cells/chamber} \times 5{,}000}{10} = \text{cells/ml}$$

8. Use this calculation to estimate the number of cells per ml for the other dilutions and then use this data to determine plating efficiency.

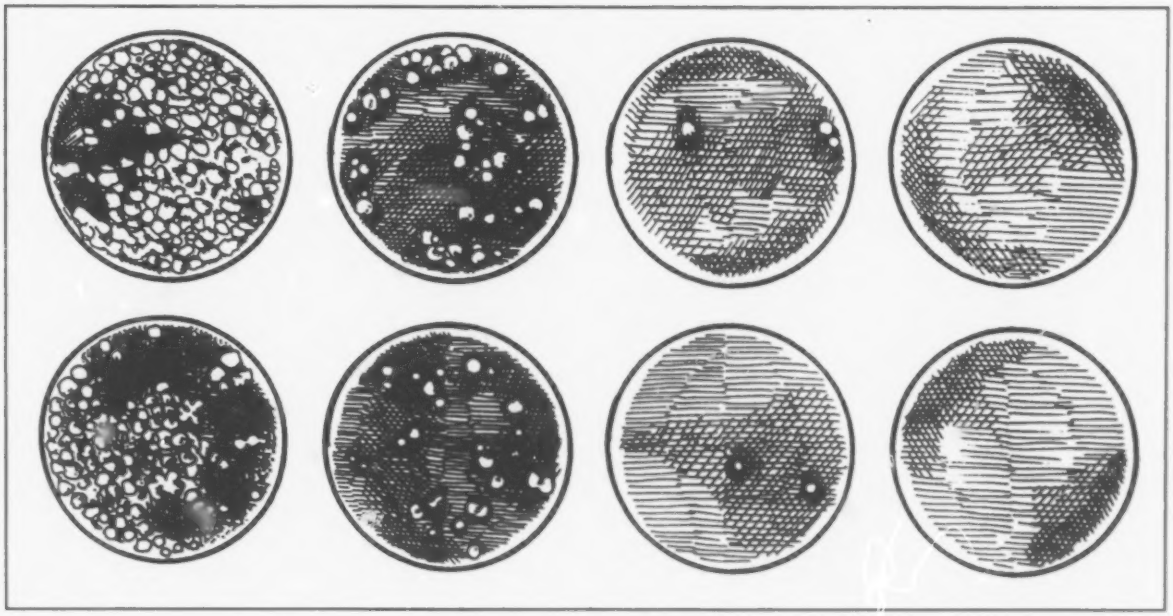


Figure 13.1. Appearance of plates at each dilution after 4 weeks of incubation. Top row is with casein hydrolysate, and bottom row is without casein hydrolysate. From left to right: undiluted, 1/10, 1/100, and 1/1,000 dilutions.

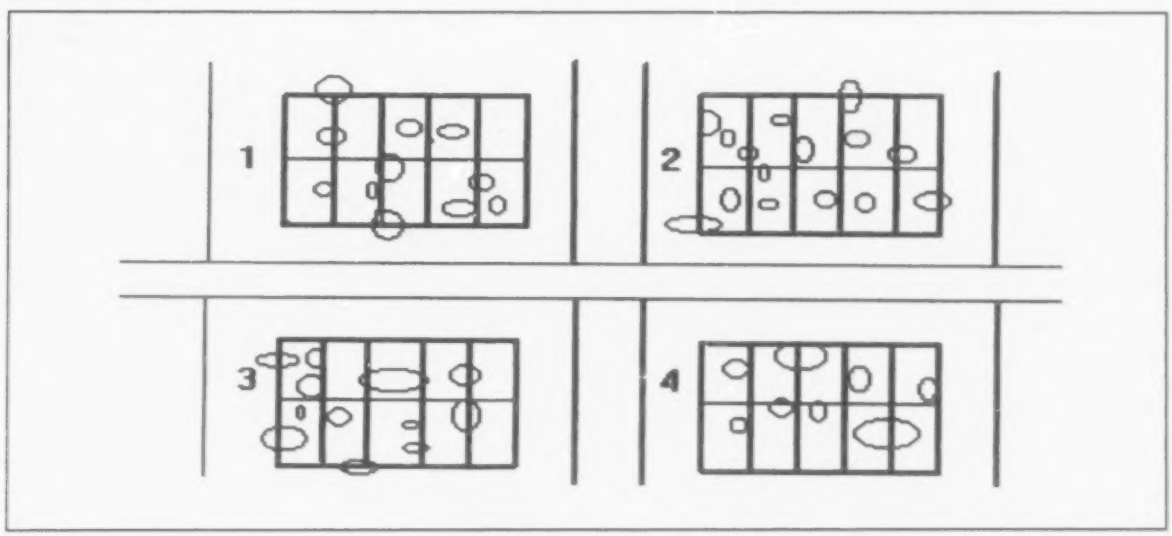


Figure 13.2. Appearance of Speirs-Levy counting chambers with plant cells.

Report 13
Sweet Potato Cell Cultures Name:

1. Record the cell and colony counts for each dilution in the following table:

Inoculation Size (SCV)	Cell Count (cell/ml)	Colony Count		Plating Efficiency	
		with CH	without CH	with CH	without CH
5 ml					
0.5 ml					
0.05 ml					
0.005 ml					

2. Is there any influence of casein hydrolysate on plating efficiency?

3. If desired, repeat the experiment four more times or collect data from four (4) additional student pairs and perform the appropriate statistical analyses.

Optional

Design a study of your own using the investigation form provided in Appendix E. Following are some suggested questions:

1. What would be the effect on plating efficiency if coconut milk were used as a supplement?
2. Would the plating efficiency for carrot cell cultures be comparable to that of sweet potato?

14. Pea Shoot Tip Cultures

Objective

To gain practice establishing and maintaining shoot tip cultures.

Background

Developing a shoot tip multiplication protocol that provides consistent results is a first step in cryopreservation and other studies; however, there can be differences in response due to genotype. Thus, there are many studies needed to define optimum conditions for each genotype. Pea seeds *(Pisum sativum)* are useful to demonstrate the technique of shoot tip culturing. They are disinfected and germinated five to seven days before the exercise and, thus, do not require long-term care. In addition, they are easy to dissect, and you can prepare as many disinfected shoot tips as needed for your experimental design.

In this exercise, the case study of Exercise 4 will be repeated, using a factorial experimental design to determine the effects of benzylaminopurine and alphanapthaleneacetic acid, alone and in combination, on shoot tip production

Materials

(per student pair)

- 1 large forceps, sterile
- 1 spatula, sterile
- 1 scalpel, sterile
- 1 300-ml beaker containing 250 ml 95% ethanol
- 5 tubes (16 mm x 100 mm) of each treatment (Table 14.1), 5 ml/tube. (Basal medium consists of Murashige and Skoog salt base with 1 mg/L thiamine HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 100 mg/L inositol, 30 g/L sucrose, 8 g/L agar.)
- 1 500-ml beaker containing 30 ml distilled water, sterile
- 30 pea seeds (*Pisum sativum* cv. Little Marvel)
- 1 250-ml beaker covered with aluminum foil for pea seed disinfection
- bleach solution, 20% (v/v), 200 ml
- 95% ethanol, 50 ml
- 6 sterile petri dishes (100 mm x 15 mm)
- 3 300-ml flasks, each containing 250 ml sterile distilled water

Additional Materials

- growth chamber set a 25° C and a 16-hour photoperiod (approximately 2,000 lux)
- incubator set at 25° C
- dissecting scope
- Jiffy® pellets
- plastic sandwich bags

Procedure

Preparation of Pea Seedlings

1. Pea seeds (*Pisum sativum* cv. Little Marvel) are disinfected by soaking in 95% ethanol for one minute followed by 20 minutes in 20% (v/v) bleach. This is best accomplished with constant stirring.

2. Then rinse the seeds three times with sterile distilled water.

3. Place 30 of these seeds in a sterile, 500-ml aluminum foil-covered beaker containing 30 ml of sterile distilled water.

4. Incubate in the dark at 25° C for five to seven days. The radicles and shoot tips now should be visible. The water in the beaker should be clear if there is no contamination.

Dissection and Testing of Shoot Tips

1. Using forceps, place one of the germinated peas in the bottom half of a sterile petri dish.

2. Hold the seedling steady with the forceps and, using the scalpel, remove the terminal 5 to 6 mm of shoot tip. This includes the apical meristem, four to five pairs of leaf primordia, and two lateral buds.

3. Transfer this shoot tip to one of the treatment tubes. Use the spatula to press the base of the tip into the agar surface. Repeat this process until all treatments have been replicated five times.

4. Incubate at 25° C with a 16-hour photoperiod (approximately 2,000 lux) for six weeks. Then, determine the average number of shoots forming per explant. A dissecting scope must be used since some of the shoots will have many new small tips in the axils of the leaves (see Figure 4.1, Exercise 4).

5. Record the data in the table provided.

Subculturing of Shoot Tips

1. Separate and transfer tips to Murashige and Skoog medium supplemented with 0.45 mg/L BA every four to five weeks for maintenance.

2. For plantlet production, transfer shoots to media without hormones for rooting. Incubate at 25° C with a 16-hour photoperiod (approximately 2,000 lux) until plantlets have developed shoots and roots. They should be one to two inches in height.

3. At this time, gently remove plantlets from the agar medium, wash off any clinging agar, and transplant to Jiffy® pellets. Enclose the pellets in plastic bags to maintain high humidity. Following establishment of plantlets in Jiffy® pellets, transfer to soil and gradually expose to normal light and humidity.

Table 14.1. Hormone treatments for shoot tip cultures.

Treatment	NAA (mg/L)	BA (mg/L)
A	0.00	0.00
B	0.00	0.45
C	0.02	0.00
D	0.02	0.45
E	0.20	0.00
F	0.20	0.45

Report 14
Pea Shoot Tip Cultures

Name:

1. Following six weeks of incubation, record the number of shoot tips forming per replicate for each treatment in the following table:

		BA (mg/L)		
		0.0	0.45	
NAA (mg/L)				
0.0	1:	4:	1:	4:
	2:	5:	2:	5:
	3:		3:	
0.02	1:	4:	1:	4:
	2:	5:	2:	5:
	3:		3:	
0.20	1:	4:	1:	4:
	2:	5:	2:	5:
	3:		3:	

2. Before performing the statistical analysis, which treatment appears to be most effective, if any? Explain.

3. Which statistical procedure(s) is (are) appropriate for analysis of the data?

4. What are the **null hypotheses** for this experiment?

5. State the **alternative hypotheses** for this experiment.

6. Perform the appropriate statistical analyses and record below.

7. Which hormone treatments, if any, influenced average number of shoot tips formed?

8. How do your results compare to those of the case study presented in Exercise 4? Should the results be exactly the same? Explain.

Optional

Design a study of your own using the investigation form provided in Appendix E. Following are some suggested questions:

1. Would the results obtained for the pea variety "Little Marvel" be applicable to other pea varieties?
2. Would the same hormone combination be best for recovery of "Little Marvel" from cryopreservation?
3. How would other media components, such as organics and inorganics, influence shoot proliferation?

15. *Cattleya* Shoot Tip Cultures

Objective

To gain practice establishing orchid shoot tip cultures.

Background

Cattleya species represent a very important crop for cut-flower growers. There has long been interest in the use of plant tissue culture techniques for propagation and further growth of these orchids (Reinert & Mohr, 1967; Scully, 1967; Lindemann et al., 1970: Ichihashi & Kako, 1973; Kusumoto, 1979a and 1979b; Mauro et al., 1994). Hormone concentrations optimum for shoot multiplication usually are different from those necessary for root or shoot biomass production. For example, Pierik and Steegmans (1972) found that high concentrations of benzylaminopurine (BA) favored protocorm and plantlet formation and also increased mean fresh weight of protocorms and shoots, but these same conditions decreased mean fresh weight of roots. Others have found that napthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) alone were essential for initiation of *Cattleya* shoot tips (Ichihashi & Kako, 1973).

In this exercise, the influence of BA and NAA, alone and in combination, on shoot tip multiplication of *Cattleya aurantiaca* will be determined.

References

Ichihashi, S. & Kako, S. (1973). Studies on clonal propagation of *Cattleya* through tissue culture methods I. Factors affecting survival and growth of shoot meristem of *Cattleya in vitro*. *Journal of the Japanese Society for Horticultural Science, 42,* 264-270.

Kusumoto, M. (1979a). Effects of combinations of growth regulators, and of organic supplements, on the growth of *Cattleya* plantlets cultured *in vitro*. *Journal of the Japanese Society for Horticultural Science, 47,* 492-501.

Kusumoto, M. (1979b). Effects of combinations of growth regulators, and of organic supplements, on the proliferation and organogenesis of *Cattleya* protocorm-like bodies cultured *in vitro*. *Journal of the Japanese Society for Horticultural Science, 47,* 502-510.

Lindemann, E.G.P., Gunckel, J.E. & Davidson, O.W. (1970). Meristem culture of *Cattleya*. *American Orchid Society Bulletin, 39,* 1002-1004.

Mauro, M., Sabapathi, D. & Smith, R.A. (1994). Influence of benzylaminopurine and alphanapthaleneacetic acid on multiplication and biomass production of *Cattleya aurantiaca* shoot explants. *Lindleyana, 9,* 169-173.

Pierik, R.L.M. & Steegmans, H.H.M. (1972). The effect of 6-benzylaminopurine on growth and development of *Cattleya* seedlings grown from unripe seeds. *Zeitschrift fuer Pflanzenphysiologie, 68,* 228-234.

Reinert, R.A. & Mohr, H.C. (1967). Propagation of *Cattleya* by tissue culture of lateral bud meristems. *Proceedings of the American Society for Horticultural Science, 91,* 664-671.

Scully, R.M., Jr. (1967). Aspects of meristem culture in the *Cattleya* alliance. *American Orchid Society Bulletin, 36,* 103-108.

Materials

(per student pair)

- 1 large forceps, sterile
- 1 spatula, sterile
- 1 scalpel, sterile
- 1 300-ml beaker containing 250 ml 95% ethanol
- 5 tubes (16 mm x 100 mm) of each treatment (Table 15.1). The basal medium consists of Murashige and Skoog salt base with 1 mg/L thiamine HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 100 mg/L inositol, 20 g/L sucrose, 2 g/L peptone, 1 g/L MES (2-[N-Morpholino]ethane sulfonic acid), and 8 g/L agar.

- 30 *Cattleya aurantiaca* Rolfe seedlings, purchased from a commercial supplier.
- 6 sterile petri dishes (100 mm x 15 mm)

Additional Materials

- growth chamber set a 25° C and a 16-hour photoperiod (approximately 2,000 lux)

Procedure

1. Using forceps, place one of the *Cattleya* seedlings in the bottom half of a sterile petri dish.

2. Hold the seedling steady with the forceps and, using the scalpel, remove the root and, if necessary, trim the leaves to provide shoot explants 2-4 mm long.

3. Transfer this shoot tip to one of the treatment tubes. Use the spatula to press the base of the tip into the agar surface. Repeat this process until all treatments have been replicated five times.

4. Incubate at 25° C with a 16-hour photoperiod (approximately 2,000 lux) for 10 weeks. Then, determine the average number of shoots forming per explant. A dissecting scope may be necessary since some of the shoots will be less than 1 mm.

5. Record the data in the table provided.

Table 15.1. Hormone treatments for *Cattleya* shoot tip cultures.

Treatment	NAA (mg/L)	BA (mg/L)
A	0.00	0.00
B	0.00	10.00
C	0.10	0.00
D	0.10	10.00
E	1.00	0.00
F	1.00	10.00

Report 15
Cattleya Shoot Tip Cultures

Name:

1. Following 10 weeks of incubation, record the number of shoot tips forming per replicate for each treatment in the following table:

	BA (mg/L)			
	0.0		10.00	
NAA (mg/L)				
0.0	1:	4:	1:	4:
	2:	5:	2:	5:
	3:		3:	
0.10	1:	4:	1:	4:
	2:	5:	2:	5:
	3:		3:	
1.00	1:	4:	1:	4:
	2:	5:	2:	5:
	3:		3:	

2. Before performing the statistical analysis, which treatment appears to be most effective, if any? Explain

3. Which statistical procedure(s) is (are) appropriate for analysis of the data?

4. What are the **null hypotheses** for this experiment?

5. State the **alternative hypotheses** for this experiment.

6. Perform the appropriate statistical analyses and record below.

7. Which hormone treatments, if any, influenced average number of shoot tips formed?

8. Are there any obvious patterns; e.g., with root growth? Explain.

Optional

Design a study of your own using the investigation form provided in Appendix E. Following are some suggested questions.

1. Would some combination of kinetin and IAA have a greater influence on production of shoot tips?
2. Would a higher concentration of BA have a greater influence on production of shoot tips?
3. What would be the influence of removing peptone from the medium? Of removing the MES?

UNIT IV – REGENERATION

Regeneration includes **somatic embryogenesis** and **organogenesis**. Embryogenesis is the process of embryo initiation and development; whereas, organogenesis is the evolution of a structure that shows natural organ form or function or both (Schaeffer, 1990). The success of many *in vitro* techniques in higher plants depends on the success of plant regeneration (Flick et al., 1983).

Organogenesis

Regeneration of tobacco (Skoog & Miller, 1957) from explants or calluses is the classical example used to demonstrate *organogenesis*. It is a good choice since effects of auxins and cytokinins on tobacco are specific and reproducible (Flick et al., 1983). High auxin and low cytokinin concentrations promote callus formation. Shoot formation occurs with low auxin and high cytokinin concentrations. Roots form with high auxin and very low cytokinin concentrations or with auxin alone. Also, tobacco callus may be obtained easily from any of several biological supply houses and subcultured to produce the quantity needed.

This "Skoog-Miller" model for tobacco callus holds for many species (Hussey, 1986). There are exceptions, though. For example, "In general, monocotyledonous species do not show a pronounced response to cytokinins but require auxins to achieve changes in the development of cultured tissues" (Meins, 1986). These exceptions allow an opportunity to bring experimentation and problem solving into the classroom.

Embryogenesis

Embryogenesis from callus growth has been obtained from many plant species (Evans et al., 1981). For carrot *(Daucus carota)* roots, disinfected explants are placed on an agar medium containing 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) to initiate callus (Ammirato, 1983). The callus is subcultured on media devoid of 2,4-D or other hormones to allow embryo development. Plantlets develop on agar media devoid of 2,4-D.

Embryogenic tissue cultures have led to the development of efficient procedures for plant regeneration of cereal crops (Vasil, 1988). In addition, somatic embryos have been used in many other protocols, including transformation studies using *Agrobacterium tumefaciens* (McGranahan et al., 1988).

References

Ammirato, P.V. (1983). Embryogenesis. In D.A. Evans, W.R. Sharp, P.V. Ammirato & Y. Yamada (Eds.), *Handbook of Plant Cell Culture, Volume I: Techniques for Propagation and Breeding*, 82-123. New York: Macmillan Publishing Company.

Evans, D.A., Sharp, W.R. & Flick, C.E. (1981). Growth and behavior of cell cultures: Embryogenesis and organogenesis. In T.A. Thorpe (Ed.), *Plant Tissue Culture Methods and Applications in Agriculture*, 45-113. New York: Academic Press.

Flick, C.E., Evans, D.A. & Sharp, W.R. (1983). Organogenesis. In D.A. Evans, W.R. Sharp, P.V. Ammirato & Y. Yamada (Eds.), *Handbook of Plant Cell Culture, Volume I: Techniques for Propagation and Breeding*, 13-81. New York: Macmillan Publishing Company.

Hussey, G. (1986). Vegetative propagation of plants by tissue culture, In M.M. Yeoman (Ed.), *Botanical Monographs, Volume 23: Plant Cell Culture Technology*, 29-66. Boston, MA: Blackwell Scientific Publications.

McGranahan, G.H., Leslie, C.A., Uratsu, S.L., Martin, L.A. & Dandekar, A.M. (1988). *Agrobacterium*-mediated transformation of walnut somatic embryos and regeneration of transgenic plants. *Biotechnology, 6*, 800-804.

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Meins Jr., F. (1986). Determination and morphogenetic competence in plant tissue culture. In M.M. Yeoman (Ed.), *Botanical Monographs, Volume 23: Plant Cell Culture Technology*, 7-25. Boston, MA: Blackwell Scientific Publications.

Schaeffer, W.I. (1990). Terminology associated with cell, tissue and organ culture, molecular biology, and molecular genetics. *In Vitro Cellular and Developmental Biology, 26*, 97-101.

Skoog, F. & Miller, C.O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symposia of the Society for Experimental Biology, 11*, 118-131.

Vasil, I.K. (1988). Progress in the regeneration and genetic manipulation of cereal crops. *Biotechnology, 6*, 397-402.

16. Somatic Embryogenesis of Carrot Cultures

Objectives

1. To gain practice initiating embryogenesis in carrot cultures.

2. To gain an appreciation of factors influencing induction of carrot embryogenesis.

Background

Kamada and Harada (1979) found that, for carrot cultures established from hypocotyl segments, duration of 2,4-dichlorophenoxyacetic acid (2,4-D) treatment influenced embryogenesis and organogenesis. For example, for explants receiving a one-day exposure to 2,4-D, followed by transfer to media devoid of hormones, 2.9% formed embryos; whereas, 61.8% formed roots. One the other hand, for explants receiving a two-week exposure to 2,4-D, followed by transfer to media devoid of hormones, 100% formed embryos; whereas, 0% formed roots.

In this exercise, the influence of exposure time to 2,4-D on organogenesis and embryogenesis using carrot taproot will be tested.

Reference

Kamada, H. & Harada, H. (1979). Studies on the organogenesis in carrot tissue cultures I. Effects of growth regulators on somatic embryogenesis and root formation. *Zeitschrift fuer Pflanzenphysiologie, 91,* 255-266.

Materials

(per student pair)

- 1 scalpel, sterile
- 1 large forceps, sterile
- 1 300-ml beaker containing 250 ml 95% ethanol
- 1 carrot taproot callus culture established as in Exercise 10, *15 weeks* since initiation
- 1 carrot taproot callus culture established as in Exercise 10, *2 weeks* since initiation
- 2 sterile petri plates (100 mm x 15 mm)
- 8 petri plates (100 mm x 15 mm), each containing 20 ml Murashige and Skoog salt base supplemented with 1 mg/L 2,4-D, 100 mg/L inositol, 0.4 mg/L thiamine HCl, 0.5 mg/L pyridoxine HCl, 0.5 mg/L nicotinic acid, 2 mg/L glycine, 1 g/L casein hydrolysate, 30 g/L sucrose, and 8 g/L agar.

Additional Materials

- parafilm
- growth chamber set at 25° C and a 16-hour photoperiod (approximately 2,000 lux)

Procedure

1. Place sterile instruments in the beaker containing the 95% ethanol.

2. Using the forceps, place callus from the *15-week carrot culture* in a sterile petri dish (100 mm x 15 mm).

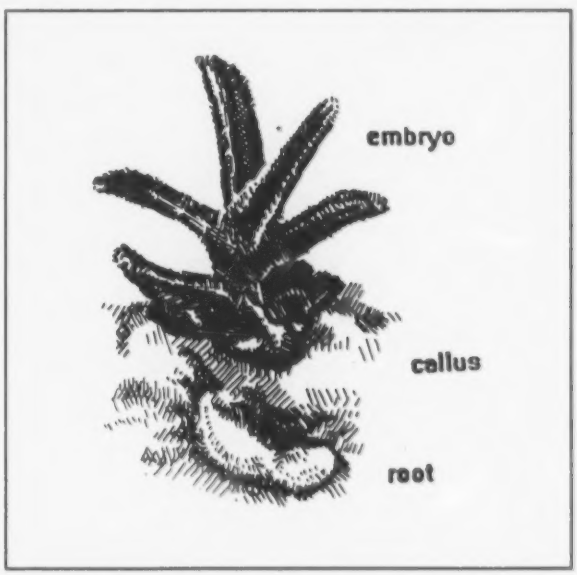

Figure 16.1. Organogenesis and embryogenesis from carrot callus cultures.

3. Divide the pieces of callus into explants about 5 mm x 5 mm to obtain a total of 10 explants.

4. Use forceps to carefully transfer five explants to each of two plates of Murashige and Skoog medium. Arrange the explants as in Figure 2.1, Exercise 2, so that they rest flat on the agar surface and do not touch.

5. Repeat Steps 2 through 4 with the *2-week-old carrot callus*. Label all plates and wrap in parafilm.

6. Incubate at 25° C for three weeks with a 16-hour photoperiod (approximately 2,000 lux).

7. Following three weeks of incubation, divide and subculture all callus onto identical media. Following an additional three weeks of incubation, examine cultures for evidence of embryogenesis or organogenesis (Figure 16.1). A dissecting scope will be necessary.

8. Record results in the table provided and answer all the questions on the report form on the next page.

Report 16
Somatic Embryogenesis of Carrot Cultures

Name:

1. Following six weeks of incubation, record the number of embryos or roots forming per explant for the 15- and 2-week callus cultures in the following table:

15-Week Callus Culture	2-Week Callus Culture

2. By observing the data, which treatment appears to have influenced embryogenesis or organogenesis, if any? Explain.

3. If desired, perform a statistical analysis. Which procedure is appropriate for analysis of the data? Record the calculations below.

a. What is the **null hypothesis** for this experiment?

b. State the **alternative hypothesis** for this experiment.

c. Which incubation period allowed maximum organogenesis or embryogenesis?

Optional

Design a study of your own using the investigation form provided in Appendix E. Following are some suggested questions:

1. What would be the influence of longer exposures to 2,4-D?
2. Would another auxin give the same or different results as 2,4-D?

17. Organogenesis of Tobacco Cultures

Objectives

1. To gain practice inducing organogenesis from tobacco cultures.

2. To understand the factors influencing induction of tobacco organogenesis.

Background

Actual results for tobacco organogenesis may not match perfectly with expected results. Root and shoot production from tobacco callus may not be as consistent as might be expected from published results (Skoog & Miller, 1957). Some of the observed variation is due to the amount of hormone present in each piece of callus at the time of transfer. This may vary depending upon the age of the culture and the actual part of the callus transferred.

For example, kinetin content will be approaching zero in cultures that are between three and four weeks old, especially in those parts of the callus furthest away from the medium (Reinert & Yeoman, 1982). Thus, these pieces of callus should have little to no kinetin present and should not grow on media containing IAA alone. This type of callus is used in bioassays for kinetin (Reinert & Yeoman, 1982). On the other hand, kinetin content in callus from younger cultures would be higher, thereby affecting callus, root, and shoot production on the same medium. This emphasizes the need for controlling the age of the culture and the actual portion of the callus transferred.

Other factors that may affect organogenesis for tobacco callus are phosphate level and concentration of casein hydrolysate. High concentrations of IAA repress bud formation. However, Skoog and Miller (1957) found that the addition of either KH_2PO_4 (400 mg/L) or casein hydrolysate (3 g/L) to the modified White's medium they used counteracted the inhibiting action of IAA. Since the medium used in this exercise is Murashige and Skoog salt base, containing 170 mg/L KH_2PO_4, greater shoot production in the presence of IAA is expected.

In this exercise, the influence of casein hydrolysate on tobacco organogenesis will be determined using Murashige and Skoog salt base.

References

Reinert, J. & Yeoman, M.M. (1982). *Plant Cell and Tissue Culture: A Laboratory Manual.* New York: Springer-Verlag.

Skoog, F. & Miller, C.O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro. Symposia of the Society for Experimental Biology, 11,* 118-131.

Materials

(per student pair)

- 1 large forceps, sterile
- 1 spatula, sterile
- 1 scalpel, sterile
- 1 300-ml beaker containing 250 ml 95% ethanol
- 5 tubes (20 mm x150 mm) of each treatment (Table 17.1) **with** casein hydrolysate. The basal medium consists of Murashige and Skoog salt base with 0.4 mg/L thiamine HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 100 mg/L inositol, 2 mg/L glycine, 1 g/L casein hydrolysate, 30 g/L sucrose, and 8 g/L agar.
- 5 tubes (20 mm x 150 mm) of each treatment (Table 17.1) **without** casein hydrolysate. The basal medium consists of Murashige and Skoog salt base supplemented as above except with no casein hydrolysate.
- 6 tobacco (*Nicotiana tabacum* cv. Wisconsin No. 38) callus cultures grown on Murashige and Skoog salt base supplemented as above except without casein hydrolysate and with 2 mg/L IAA and 0.03 mg/L kinetin, four weeks since the last subculture.

- 6 sterile petri dishes (100 mm x 15 mm)

Additional Materials

- growth chamber set at 25° C and a 16-hour photoperiod (approximately 2,000 lux)

Procedure

1. Tobacco *(Nicotiana tabacum)* callus is placed in a sterile petri dish (100 mm x 15 mm) and sliced into pieces approximately 5 mm x 5 mm. Five slices should be obtained from a 4-week-old culture.

2. For testing, place each piece into one of the treatment tubes. Inoculate five tubes of each treatment and incubate at 25° C with a 16-hour photoperiod (approximately 2,000 lux) for six weeks.

3. Record results in the table provided on the report form and answer all questions.

Table 17.1. Tobacco callus hormone treatments.

Treatment	IAA (mg/L)	Kin (mg/L)
A	2.00	0.20
B	0.00	1.00
C	3.00	0.00

Report 17
Organogenesis of Tobacco Cultures Name:

1. Following six weeks of incubation, record the percentage of explants showing callus, shoots, or roots formed for each treatment in the following table:

Hormones	With Casein Hydrolysate	Without Casein Hydrolysate
2.00 mg/L IAA 0.20 mg/L Kinetin		
1.00 mg/L Kinetin		
3.00 mg/L IAA		

2. On the media without casein hydrolysate, is there an obvious hormone concentration for root production? For shoot production? For callus production?

3. What effect did casein hydrolysate have on the above relationships between hormone concentration and organogenesis?

4. If desired, repeat the experiment five times or collect data from other student pairs and perform the appropriate statistical analysis.

5. Is your conclusion the same after analyzing the data statistically?

Optional

Design a study of your own using the investigation form provided in Appendix E. Following are some suggested questions:

1. What would be the influence of varying other media components, such as carbohydrate source?
2. Would more consistent results be obtained if only callus from the outer portion were used?

18. Organogenesis of Sweet Potato Root Explants

Objective

To gain an understanding of the factors influencing induction of organogenesis in different species.

Background

Roots and shoots may be initiated directly from sweet potato root explants (Gunckel et al., 1972). Sweet potatoes *(Ipomoea batatas)* belong to the morning glory family, the Convolvulaceae. Sweet potatoes are commonly sold as "yams" in the grocery store; however, sweet potatoes are dicots, and the true yams are monocots, belonging to the genus *Dioscorea.* In this exercise, the application of the Skoog-Miller model of organogenesis to sweet potato roots will be tested.

Reference

Gunckel, J.E., Sharp, W.R., Williams, B.W., West, W.C. & Drinkwater, W.O. (1972). Root and shoot initiation in sweet potato explants are related to polarity and nutrient media variations. *Botanical Gazette, 133,* 254-262.

Materials

(per student pair)

- 1 scalpel, sterile
- 1 large forceps, sterile
- 1 utility tongs, sterile
- 1 cork borer (5 or 6 mm in diameter) with punch, sterile
- 1 300-ml beaker containing 250 ml 95% ethanol
- 1 sweet potato root
- 3 300-ml flasks, each containing 250 ml sterile distilled water
- 1 600-ml beaker, covered with aluminum foil and sterilized
- 6 sterile petri plates (100 mm x 15 mm)
- bleach solution, 20% (v/v), 500 ml
- 5 tubes (20 mm x 150 mm) of each treatment (Table 18.1) with casein hydrolysate. The basal medium consists of Murashige and Skoog salt base with 0.4 mg/L thiamine HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 100 mg/L inositol, 2 mg/L glycine, 1 g/L casein hydrolysate, 30 g/L sucrose, and 8 g/L agar.
- 5 tubes (20 mm x 150 mm) of each treatment (Table 18.1) without casein hydrolysate. Murashige and Skoog salt base is supplemented as above except with no casein hydrolysate.

Additional Materials

- vegetable scraper
- kitchen knife
- growth chamber set at 25° C and a 16-hour photoperiod (approximately 2,000 lux)

Procedure

Sweet Potato Root Disinfection

1. Sweet potatoes *(Ipomoea batatas)* are scrubbed under running tap water to remove residual soil.

2. Remove the skin using a vegetable scraper and then cut the potato transversely into slices about 15-mm thick. Each student pair will need three (3) slices.

3. Disinfect the slices in an aluminum foil covered beaker filled with 20% (v/v) bleach solution for 10 minutes. Rinse three times using sterile distilled water.

Explant Preparation

1. Place sterile instruments in the beaker containing the 95% ethanol.

2. Using the utility tongs, place each slice in a separate sterile petri dish (100 mm x 15 mm).

3. Hold the slice steady with the tongs. Make borings with the cork borer parallel to the vertical axis of the tissue slice. Avoid including any of the side surface from the slice since this was damaged by the disinfectant.

4. Push the cork borer completely through the slice. Hold the slice steady with the tongs to remove the cork borer. The boring will remain inside the borer.

5. Plunge the boring into a sterile petri dish using the cork borer punch.

6. Repeat this process to obtain 10 borings from each slice, for a total of 30 borings.

7. Use a scalpel to remove about 2 mm of tissue from each end of the borings. Cylinders 10 mm in length should remain.

Testing

1. For testing, place each piece into one of the treatment tubes. Inoculate five tubes of each treatment and incubate at 25° C with a 16-hour photoperiod (approximately 2,000 lux) for 11 weeks.

2. Record results in the table provided on the report page and answer all questions.

. . . . . .

Table 18.1. Sweet potato explant hormone treatments.

Treatment	IAA (mg/L)	Kin (mg/L)
A	2.00	0.20
B	0.00	1.00
C	3.00	0.00

Report 18
Organogenesis of Sweet Potato Root Explants

Name:

1. Following 11 weeks of incubation, record the percentage of explants showing callus, shoots, or roots formed for each treatment in the following table:

Hormones	With Casein Hydrolysate	Without Casein Hydrolysate
2.00 mg/L IAA 0.20 mg/L Kinetin		
1.00 mg/L Kinetin		
3.00 mg/L IAA		

2. On the media without casein hydrolysate, is there an obvious hormone concentration for root production? For shoot production? For callus production?

3. What effect did casein hydrolysate have on the above relationships between hormone concentrations and organogenesis?

4. How do these results compare to those for tobacco? (See Exercise 17.)

5. Does the Skoog-Miller model apply to sweet potato explants?

Optional

Design a study of your own using the investigation form provided in Appendix E. Following are some suggested questions:

1. What would be the influence of varying other media components, such as carbohydrate source?
2. Would variety of sweet potato influence results?

19. Organogenesis of African Violet Leaf Explants

Objective

To gain an appreciation of the factors influencing induction of organogenesis in different species.

Background

Explants of African violet *(Saintpaulia ionantha)* leaves will form plantlets when placed on appropriate media. In addition to an auxin, such as indole-3-acetic acid (IAA), and a cytokinin, such as kinetin, which are necessary for tobacco organogenesis, adenine sulfate is added. The adenine sulfate functions in the induction of buds for African violet.

In this exercise, initiation of African violet plantlets from leaf explants on media containing adenine sulfate will be compared to media without adenine sulfate.

Materials

(per student pair)

- 1 scalpel, sterile
- 1 large forceps, sterile
- 1 300-ml beaker containing 250 ml 95% ethanol
- 1 African violet plant
- 3 300-ml flasks, each containing 250 ml sterile distilled water
- 1 600-ml beaker, covered with aluminum foil and sterilized
- 2 sterile petri plates (100 mm x 15 mm)
- bleach solution, 20% (v/v), 500 ml
- 10 tubes (20 mm x 150 mm) containing Murashige and Skoog salt base with 0.4 mg/L thiamine HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 100 mg/L inositol, 170 mg/L $NaH_2PO_4.H_2O$, 2mg/L IAA, 2 mg/L kinetin, 80 mg/L adenine sulfate, 30 g/L sucrose, and 8 g/L agar.
- 10 tubes (20 mm x 150 mm) containing Murashige and Skoog salt base supplemented as above except with no adenine sulfate.

Additional Materials

- liquid dish detergent
- growth chamber set at 25° C and a 16-hour photoperiod (approximately 2,000 lux)

Procedure

African Violet Leaf Disinfection

1. Place sterile instruments in the 300-ml beaker containing 95% ethanol.

2. Remove the younger leaves from the center portion of an African violet *(Saintpaulia ionantha)* plant purchased from a grocery store. Each pair of students will use four leaves.

3. Disinfect the slices in an aluminum foil-covered beaker filled with 20% (v/v) bleach solution to which one drop of liquid dish detergent is added. Swirl the leaves in this solution every few minutes for 10 minutes. Rinse three times using sterile distilled water.

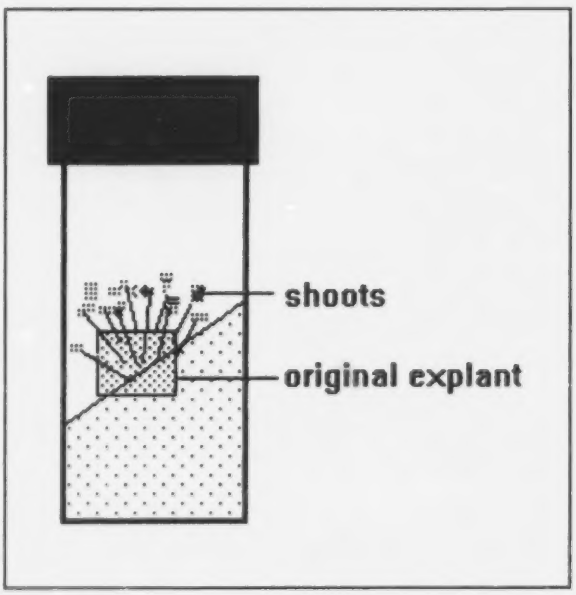

Figure 19.1. African violet plantlets.

Explant Preparation

1. Using the forceps, place one leaf, with the lower surface facing up, in a sterile petri dish (100 mm x 15 mm).

2. Hold the leaf steady with the forceps. Use the scalpel to cut and remove the outer edges of the leaf blade, leaving the center portion of the blade, including the midvein, about 5-mm wide. Cut this transversely into explants about 5-mm wide for a total of five explants from each leaf.

3. Transfer the explants to each treatment tube until all 10 tubes for each treatment have been inoculated. Press each section about one-fourth into the medium.

4. Culture in a growth chamber at 25° C with a 16-hour photoperiod (approximately 2,000 lux) for eight weeks. At this point, plantlets should be visible (Figure 19.1 on the previous page). Record your observations in the table provided and answer the questions on the report sheet.

Report 19
Organogenesis of African Violet Leaf Explants

Name:

1. Following eight weeks of incubation, record the percentage of explants showing callus, shoots, or roots formed for each treatment in the following table:

Hormone	With Adenine Sulfate	Without Adenine Sulfate
2 mg/L IAA 2 mg/L Kinetin		

2. Is adenine sulfate necessary for plantlet production?

3. If desired, repeat the experiment or collect data from additional student pairs and perform the appropriate statistical analysis.

Optional

Design a study of your own using the investigation form provided in Appendix E. Following are some suggested questions:

1. What would be the influence of varying other media components, such as carbohydrate source? Or casein hydrolysate?
2. Would other hormone combinations, such as NAA and BA, give different results?

UNIT V – PREPARATION OF TISSUE CULTURE MEDIA

Plant tissue culture is the growing of isolated plant parts aseptically on appropriate media (Steward, 1983); however, it is not easy to define "appropriate media." Plant tissue culture media generally contain a mixture of inorganic salts (macronutrients and micronutrients), vitamins, an organic carbon source (sucrose or glucose), and plant growth regulators (Gamborg et al., 1976). Agar is added if a solid medium is desired.

To the above ingredients, some authors have added compounds such as amino acids, amides, casein hydrolysate, peptone, malt extract, or yeast extract to provide an organic nitrogen source and stimulate growth (Gamborg et al., 1976; George et al., 1987). In addition, for some applications, unusual sugars, long-chain alcohols, organic acids, buffers, coconut milk, juices of plants, banana homogenate, or activated charcoal have been used (George et al., 1987; George et al., 1988; Ammirato, 1983).

Inorganic Salts

The **macronutrients** provide the major ions needed by plants, which include nitrogen, phosphorous, potassium, calcium, magnesium, and sulphur (George et al., 1988). The **micronutrients** supply the minor nutrients needed by plants and include the elements iron, manganese, zinc, boron, copper, cobalt, and molybdenum (George et al., 1988). Although numerous formulations have been developed to supply these essential elements, two widely used ones are Murashige and Skoog Salt Base (Murashige & Skoog, 1962) and Gamborg, Miller and Ojima Salt Base (Gamborg et al., 1968). These mixtures (Tables V.1 and V.2) are commercially available or can be prepared in the classroom. They represent an improvement in earlier formulations since their concentrations of inorganic nutrients should eliminate the need for complex organic supplements for many plant cells in culture (Gamborg et al., 1976).

Examination of these formulations reveals that sodium, iodine, and the chloride ion are also provided. These are not classed as essential elements but may be necessary in specific situations. For example, sodium appears to be essential to salt-tolerant plants that have a C_4 or crassulacean acid metabolism (George et al., 1988).

Vitamins

Thiamine, inositol, nicotinic acid, and pyridoxine are four commonly used vitamins for plant tissue culture media; however, none of these has been demonstrated to be essential for all systems (George et al., 1988). The need for these vitamins should be determined experimentally.

Organic Carbon Source

The requirement for an organic carbon source is usually satisfied by sucrose (Murashige, 1974). Other carbohydrates have been used, but these may influence your results. For example, Welander et al. (1989) tested the effects of sucrose, glucose, fructose, mannitol, and sorbitol on *in vitro shoot* multiplication in *Syringa, Alnus,* and *Malus.* They found that carbon source influenced the percentage of explants forming shoots, the number of shoots per explant, the number of separable nodes per shoot, and shoot length. As another example, Tal et al. (1982) determined that both sugar concentration and sugar type influenced production of diosgenin.

Plant Growth Regulators

Two major categories of plant growth regulators that may be included in plant tissue culture media are auxins and cytokinins. Some commonly used auxins include 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and naphthaleneacetic acid (NAA). Auxins are thought to control a number of processes, including cell elongation, induction of adventitious root formation, and induction of disorganized growth (Jacobsen, 1983). The

auxin 2,4-D is very effective for inducing callus in many plants, although higher concentrations are needed for this purpose for monocots (Vasil, 1988).

Auxins may be used alone or in combination with a cytokinin. Frequently used cytokinins include adenine sulfate, kinetin, 6-benzylaminopurine (BA), zeatin, and (2-isopentenyl)-adenine (2iP). Cytokinins stimulate cell division and promote shoot morphogenesis (Jacobsen, 1983; Flick et al., 1983). For each species, the ratio of

Table V.1. Murashige and Skoog Salt Base (Carolina Biological Supply Company, 1986).

Macronutrient Components (mg/L)		
NH_4NO_3		1650.000
KNO_3		1900.000
$CaCl_2$		333.00
$MgSO_4$		181.000
KH_2PO_4		170.000
Micronutrient Components		
FeNaEDTA		36.700
H_3BO_3		6.200
$MnSO_4.H_2O$		16.900
$ZnSO_4.7H_2O$		8.600
KI		0.830
$Na_2MoO_4.2H_2O$		0.250
$CuSO_4.5H_2O$		0.025
$CoCl_2.6H_2O$		0.025
	Total	4303.530

Table V.2. Gamborg, Miller and Ojima Salt Base (Carolina Biological Supply Company, 1986).

Macronutrient Components (mg/L)		
$(NH_4)_2SO_4$		134.000
$CaCl_2.2H_2O$		150.000
$MgSO_4.7H_2O$		250.000
KNO_3		2500.000
$NaH_2PO_4.H_2O$		150.000
Micronutrient Components		
H_3BO_3		3.000
$CoCl_2.6H_20$		0.025
$CuSO_4.5H_2O$		0.025
$FeSO_4.7H_2O$		27.800
$MnSO_4.H_2O$		10.000
KI		0.750
Na_2EDTA		37.300
$Na_2MoO_4.2H_2O$		0.250
$ZnSO_4.7H_2O$		2.000
	Total	3265.150

auxin to cytokinin in the medium is varied to determine optimum concentrations for callus, root, shoot, or embryo formation. A two-factor experimental design (see Exercise 4) is useful, and perhaps mandatory, for this purpose.

Other categories of hormones that may be important in plant tissue cultures are the gibberellins and ethylene. Gibberellic acid (GA_3) has been used for shoot tip cultures of potato (Goodwin et al., 1980; Creissen & Karp, 1985); however, Creissen and Karp (1985) found that ethylene built up in unvented cultures of potato, resulting in poor shoot development.

Organic Nitrogen

Inorganic salts, an organic carbon source, vitamins, and growth regulators should be sufficient for the culturing of plant tissues under most experimental conditions (Gamborg et al., 1976). Organic nitrogen sources may be used for specific purposes. For example, casein hydrolysate is used for tobacco cultures (Flick et al., 1983). Also, peptone accelerates regeneration of protocorm-like bodies from leaf segments of *Rhynchostylis retusa* (Vij et al., 1984).

Other Supplements

Unusual sugars and long-chain alcohols are used for the culturing of protoplasts to regulate the osmotic potential of the medium (George et al., 1988). Organic acids have been used to provide a buffering effect (George et al., 1988) or to promote growth of cells and protoplasts at low inoculation densities (Kao & Michayluk, 1975). Activated charcoal has been used for embryogenesis (Ammirato, 1983) and shoot tip cultures (Hu & Wang, 1983). Inclusion of these supplements for specific systems should be based upon prior research and experimentation.

Selection of Media

Selection of specific medium and supplements depends upon the plant species and the intended use of the culture (George et al., 1988). Start with media and supplements suggested in published reports. If there is no prior work for the selected plant, George et al. (1988) have provided generalized formulations for cultures.

Media Preparation and Storage

Some plant tissue culture media may be purchased premade from biological supply houses. For other applications, it may be desirable to purchase the basal salts, vitamins, hormones, and other additives as separate items and prepare the media in the classroom. This is the approach used in this book. Moreover, for more advanced applications, it may be best to prepare the basal salt mixture also. Guidelines for this process are provided in George et al. (1988).

Another important aspect of tissue culture media is the method of sterilization. The easiest and a frequently used procedure is to dispense media into flasks or tubes and autoclave for 15 minutes at 121° C. This is the method used for most of the experiments in this book. Some components of plant tissue culture media, however, may be degraded upon autoclaving. For example, sucrose is partially hydrolyzed to glucose and fructose with autoclaving (Ball, 1953). The resultant medium contains a mixture of these sugars. As noted previously, the organic carbon source may influence results. Some other components that may be changed or destroyed in autoclaved media are IAA, GA_3, and thiamine (Gamborg et al., 1976; Goodwin et al., 1980).

To avoid degradation of components, liquid media ideally should be sterilized using a 0.22 micrometer filter. For solid media, a double strength agar mixture must be autoclaved. The salt mixture, sucrose, and all other ingredients are made to double strength and filter sterilized. This filter-sterilized solution then is added to the autoclaved agar and dispensed into sterile containers. **Store** prepared media at 5° C whichever method is used.

In this unit, the preparation of medium will be introduced. In Exercise 20, the medium will be sterilized using autoclaving. In Exercise 21, the medium will be prepared using the same ingredients; however, it will be sterilized using filtration. The influence of both procedures on the final composition of the medium will be evaluated in Exercise 22.

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20. Autoclave-Sterilized Media

Objectives

1. To gain practice preparing media using an **autoclave**.

2. To gain practice evaluating the appropriateness of two protocols for media preparation.

Background

Many aspects associated with media preparation are used as a matter of convenience or habit. Sterilization of media by autoclaving is much easier than sterilization by filtration. Most workers choose to sterilize their media by autoclaving at 121° C and 15 psi for 15 minutes or longer, depending on the volume of medium per container. There may be a problem with this since a number of media components are thermolabile and may be partially or completely decomposed by autoclaving. If this practice is investigated, instead of just repeated, it might lead to an improvement in a particular research system. In this exercise, media will be prepared using an autoclave for sterilization. In the next exercise, the same media will be prepared using filtration as the means for sterilization. The growth of tobacco callus on these media will be compared in Exercise 22.

Materials

- 1 package Murashige and Skoog salt base
- 1 2-liter Erlenmeyer flask
- 1 magnetic spinbar
- 1 hot plate-stirrer
- distilled water, 1 L
- sucrose, 20 g
- inositol, 100 mg
- 2 ml 1-mg/ml IAA stock solution (see Appendix A)
- 1 ml vitamin and glycine stock solution (see Appendix A)
- 7.5 ml 0.001-mg/ml kinetin stock solution (see Appendix A)
- Difco Bacto agar, 8 g
- 10 ml 1 N HCl
- 10 ml 1 N NaOH
- 4 500-ml Erlenmeyer flasks, cotton-plugged
- 40 sterile petri plates (15 mm x 100 mm)

These materials will provide enough media for 10 pairs of students.

Additional Materials

- pH paper
- 1-ml pipets and mechanical pipetting device
- weigh boats, spatulas, and balance
- 1-liter graduate cylinder
- autoclave gloves

Procedure

1. Pour 900 ml distilled water into a 2-liter flask.

2. Add the Murashige and Skoog salt base.

3. Mix the solution using the magnetic spinbar and hot plate-stirrer.

4. Add 20 g sucrose and 100 mg inositol. Using the stock solutions, add 0.5 mg nicotinic acid, 0.5 mg pyridoxine HCl, 0.4 mg thiamine HCl, 2 mg glycine, and 2 mg IAA.

5. Adjust the pH of the medium to 5.7 using 1 N HCl or NaOH and pH paper.

6. Add 8 g agar and bring the volume to 1 liter with distilled water.

7. Heat until clear.

8. Pour 250 ml into each 500-ml Erlenmeyer flask. Now, using the 0.001 mg/ml (1 µg/L) kinetin stock solution, adjust the kinetin content of each flask as follows:

   flask 1 — 0 µg/L
   do not add stock solution

flask 2 — 5 µg/L
add 1.25 ml stock solution

flask 3 — 10 µg/L
add 2.5 ml stock solution

flask 4 — 15 µg/L
add 3.75 ml stock solution

9. Label the flasks and then plug with nonabsorbent cotton and autoclave for 20 minutes at 121° C.

10. Following autoclaving, pour 25 ml medium into each 100 mm x 15 mm petri dish. Label each dish as to kinetin content.

11. Allow to cool to room temperature and store, wrapped in plastic, at 5° C until used.

12. Use these media for Exercise 22. Record results on the report sheet for that exercise. (See page 126.)

21. Filter-Sterilized Media

Objectives

1. To gain practice using **filtration** as a means for sterilization of media.

2. To gain practice evaluating the appropriateness of two protocols for media preparation.

Background

This exercise is an extension of Exercise 20. Although it is more tedious to prepare media using filtration, there will be less destruction of thermolabile components. Thus, the same media will be prepared as in Exercise 20 except that only the agar portion will be autoclaved.

Materials

- 1 package Murashige and Skoog salt base
- 2 1-liter Erlenmeyer flasks
- 1 magnetic spinbar
- 1 hot plate-stirrer
- distilled water, 1 L
- sucrose, 20 g
- inositol, 100 mg
- 2 ml 1-mg/ml IAA stock solution (see Appendix A)
- 1 ml vitamin and glycine stock solution (see Appendix A)
- 7.5 ml 0.001-mg/ml kinetin stock solution (see Appendix A)
- Difco Bacto agar, 8 g
- 10 ml 1 N HCl
- 10 ml 1 N NaOH
- 4 500-ml sterile Erlenmeyer flasks, cotton-plugged
- 40 sterile petri plates (15 mm x 100 mm)
- 1 0.22 micrometer filtration unit, 500 ml capacity

These materials will provide enough media for 10 pairs of students.

Additional Materials

- pH paper
- 1-ml pipets and mechanical pipetting device
- weigh boats, spatulas, and balance
- 1-liter graduate cylinder
- vacuum pump
- autoclave gloves

Procedure

Double Strength Agar

1. Place 8 g Difco Bacto agar into a 1-liter Erlenmeyer flask.

2. Bring the volume to 500 ml with distilled water. Autoclave for 20 minutes at 121° C.

3. After autoclaving, swirl the flask evenly to dispense the agar and then allow to cool to 60° C.

Double Strength Murashige and Skoog Medium

1. Pour 400 ml distilled water into a 1-liter flask.

2. Add the powdered Murashige and Skoog salt base. Stir using the spinbar and magnetic hot plate.

3. Next, add the 100 mg inositol and 20 g sucrose.

4. Using the stock solutions, add 2 mg glycine, 0.5 mg nicotinic acid, 0.5 mg pyridoxine HCl, 0.4 mg thiamine HCl, and 2 mg IAA.

5. Adjust the pH of the medium to 5.7 using 1 N HCl or NaOH and bring the volume to 500 ml.

6. Sterilize by filtration (0.22 micrometer filter) with the aid of the vacuum pump.

Experimental Treatments

1. Mix the autoclaved double strength agar with the filter-sterilized double strength Murashige and Skoog medium.

2. Pour 250 ml medium into each sterile 500-ml flask

and adjust the hormonal concentrations as in Exercise 20.

3. Mix thoroughly and then dispense 25 ml into each sterile 15 x 100 mm petri dish. Label the dishes and allow to cool to room temperature. Store, wrapped in plastic, at 5° C until used.

4. Use these media for Exercise 22. Record results using the report sheet for that exercise.

UNIT VI – CELL CULTURE APPLICATIONS

Once basic techniques have been mastered, plant tissue cultures are used in a number of applications, such as bioassays (Rogozinska et al., 1964), production of secondary products (Schmauder et al., 1985), isolation of mutants (Chaleff & Parsons, 1978), induction of polyploidy (Chen & Goeden-Kallemeyn, 1979), and protoplast isolation, growth and fusion studies (Kao et al., 1974; Grosser & Collins, 1984).

Bioassays

Tobacco callus and soybean callus cultures may be used in assays for quantitative testing of kinetin and kinetin-like substances (Rogozinska et al., 1964). Prior to assay, the callus is subcultured on a rapid growth, low kinetin medium. For example, for *Nicotiana tabacum* var. Wisconsin 38, callus is subcultured on Murashige and Skoog medium with 2.0 mg/L IAA and only 0.03 mg/L kinetin (Rogozinska et al., 1964). Assay results show that growth is in linear proportion to the concentration of kinetin from 1 to 15 µg/L.

Secondary Products

Plant cell and organ cultures can produce a wide range of phytochemicals. Efforts to utilize these cultures as an alternative to agricultural production of phytochemicals have focused on a number of approaches to the problem. One approach is exploitation of cell cultures using bioreactors for mass cultivation (Scragg, 1992) or using immobilized cell systems (Williams & Mavituna, 1992). Another approach is use of organized cultures, such as hairy root cultures (Hamill et al., 1987; Fowler & Stafford, 1992), multiple shoot cultures (Heble, 1985), and embryogenic cultures (Ozeki & Komamine, 1985).

For each of these, investigations focus on maximum stimulation of phytochemicals by quantitative and qualitative manipulation of cultural conditions. These include manipulation of media components such as carbohydrates, micronutrients, macronutrients, and growth regulators and the use of fungal and chemical elicitors (Fowler & Stafford, 1992; DiCosmo & Misawa, 1985). Industrial scale production has been reached for shikonin, ginseng biomass, and berberine (Scragg, 1992).

Isolation of Protoplasts

Protoplasts possess all components of a plant cell excluding the cell wall. Leaves from pot-grown plants may be used as a source of cells on *in vitro* grown plantlets (Negrutin & Mousseau, 1980; Smith & McCown, 1983). The cell wall is removed using cell wall degrading enzymes such as cellulases, hemicellulases, and pectinases (Evans & Bravo, 1983). Protoplasts then may be used in cell line selection systems (Dix, 1986), in somoclonal variation studies (Creissen & Karp, 1985), in transformation systems (Draper et al., 1986), and fusion experiments for production of hybrids (Evans & Bravo, 1983; Sala et al., 1985). Plant regeneration from protoplasts can be improved using a feeder layer of nurse cells (Rhodes et al., 1988).

In this unit, tobacco callus will be used to bioassay cytokinin concentrations in autoclaved and filter sterilized media (Exercise 22). In Exercise 23, the induction of phytoalexin compounds in sweet potato suspension cultures will be studied, and in Exercise 24, a bioassay will be used for their detection. Finally, in Exercises 25 and 26, two protocols for isolation of protoplasts from leaf tissues will be presented.

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22. Bioassay of Cytokinins Using Tobacco Callus

Objective

To gain experience in using a tissue culture system for the assay of a biologically active compound.

Background

If tobacco callus is grown on Murashige and Skoog medium containing 2.0 mg/L IAA and 0.03 mg/L kinetin and then subcultured on media containing from 1 to 15 µg/L kinetin, increases in fresh and dry weights should be linear in proportion to the concentration. High yields are obtained up to 50 µg/L, but with higher levels of kinetin, the yields decrease (Rogozinska, 1964). This makes this system useful as a bioassay for kinetin and similarly active substances.

In this exercise, since cytokinin activity may vary depending upon the method of media preparation (Rogozinska et al., 1964), the activity of autoclaved kinetin will be compared to filter sterilized kinetin on tobacco callus growth.

Reference

Rogozinska, J.H., Helgeson, J.P. & Skoog, F. (1964). Tests for kinetin-like growth promoting activities of triacanthine and its isomer, 6-(y,y-dimethylallylamino)-purine. *Physiologia Plantarum, 17,* 165-176.

Materials

(per student pair)

- 1 large forceps, sterile
- 1 spatula, sterile
- 1 scalpel, sterile
- 1 300-ml beaker containing 250 ml 95% ethanol
- 1 plate (100 mm x 15 mm) of each treatment with autoclave sterilized kinetin from Exercise 20.
- 1 plate (100 mm x 15 mm) of each treatment with filter sterilized kinetin from Exercise 21.
- 2 tobacco (*Nicotiana tabacum* cv. Wisconsin No. 38) callus cultures growing on Murashige and Skoog medium containing 2.0 mg/L IAA and 0.03 mg/L kinetin and three weeks since the last subculture.
- 2 sterile petri dishes (100 mm x 15 mm)

Additional Materials

- analytical balance
- sterile petri dishes (60 mm x 15 mm) for weighing
- growth chamber set at 25° C with a 16-hour photoperiod (approximately 2,000 lux)
- parafilm

Procedure

1. Tobacco *(Nicotiana tabacum)* callus is placed into a sterile petri dish (100 mm x15 mm) and sliced into pieces weighing approximately 50 mg. It should be possible to obtain 5-10 slices from each original callus.

2. Using the sterile 60 mm x 15 mm petri dishes, aseptically weigh each piece to the nearest milligram (mg) and record in the table provided.

3. For testing, place one piece into each treatment plate, labeling plates to note weights of callus.

4. Inoculate one plate of each treatment, wrap in parafilm, and incubate at 25° C with a 16-hour photoperiod (approximately 2,000 lux) for four weeks.

5. Following four weeks incubation, reweigh the callus pieces to determine fresh weights.

6. Record results in the table provided on the next page and answer all questions.

Report 22
Bioassay of Cytokinins Using Tobacco Callus

Name:

1. Record fresh weights initially and following four weeks of incubation for each treatment in the table below:

Concentration of Kinetin	Initial Fresh Weight (mg) Autoclaved Kinetin	4-Week Fresh Weight (mg) Autoclaved Kinetin	Initial Fresh Weight (mg) Filtered Kinetin	4-Week Fresh Weight (mg) Filtered Kinetin
0 µg/L				
5 µg/L				
10 µg/L				
15 µg/L				

2. Plot the increase in fresh weight for each treatment in the graph below:

3. Was there a difference in tobacco callus growth for autoclaved kinetin compared to filter sterilized kinetin?

4. If statistical analysis is desired, repeat the experiment or collect data from five student pairs and perform the appropriate test below.

Optional

Design a study of your own using the investigation form provided in Appendix E. Following are some suggested questions:

1. Would using another cytokinin, such as BA, yield comparable results?
2. Would adding coconut milk as a supplement influence results?

23. Induction of Phytoalexins in Sweet Potato Suspension Cultures

Objective

To gain experience using a tissue culture system for the induction of secondary metabolites.

Background

Phytoalexins are antimicrobial compounds normally present in low concentrations in intact plants. These compounds will be produced in higher concentrations in plants in response to stressors such as fungal infection or chemicals. For example, sweet potatoes *(Ipomoea batatas)* will produce phytoalexins known as "furano-terpenes" when infected by *Ceratocystis fimbriata* or in response to chemicals such as $HgCl_2$ (Natori et al., 1981; Miller & Maxwell, 1983). Oba and Uritani (1979) tested application of this idea using plant tissue culture techniques. They determined that yeast extract elicited phytoalexin production in sweet potato suspension cultures.

In this exercise, the influence of varying yeast extract concentrations on biosynthesis of phytoalexins will be studied using sweet potato suspension cultures.

References

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Oba, K. & Uritani, I. (1979). Biosynthesis of furano-terpenes by sweet potato cell culture. *Plant & Cell Physiology, 20,* 819-826.

Materials

(per student pair)

- 4 sweet potato *(Ipomoea batatas)* suspension cultures, initiated using the techniques described in Exercise 12, growing in 250-ml Erlenmeyer flasks containing 50 ml of Gamborg's B-5 medium supplemented with 20 g/L sucrose and 1 mg/L 2,4-D, two weeks since the last subculture
- Gamborg's B-5 medium supplemented with 20 g/L sucrose, 1 mg/L 2,4-D, 200 ml
- Gamborg's B-5 medium supplemented with 20 g/L sucrose, 1 mg/L 2,4-D, and 2.5 g/L yeast extract, 200 ml
- Gamborg's B-5 medium supplemented with 20 g/L sucrose, 1 mg/L 2,4-D, and 5.0 g/L yeast extract, 200 ml
- Gamborg's B-5 medium supplemented with 20 g/L sucrose, 1 mg/L 2,4-D, and 10.0 g/L yeast extract, 200 ml
- chloroform:methanol (50:50, v/v) solvent, 500 ml

Additional Materials

- sterile 50-ml centrifuge tubes
- rotary shaker
- Whatman 1PS phase separator paper
- 200-ml glass flasks with screw caps
- glass funnels
- 100-ml graduate cylinders
- 100-ml beakers
- fume hood
- chromatography tank with 100 ml solvent (toluene:ethyl acetate [80:20, v/v])
- silica gel thin layer plates, 20 cm x 20 cm
- spray reagent [2% paradimethylaminobenzaldehyde in concentrated HCl:H_2O (50:50, v/v)]
- capillary tubes
- chloroform solvent
- drying oven

Procedure

Testing Influence of Yeast Extract

1. Transfer 50 ml of each established sweet potato suspension culture to a sterile 50-ml centrifuge tube.

2. Allow the cells to settle for 30 minutes. Determine the settled-cell volume (see Exercise 12). Use this as a guide to set up four tubes with a final inoculation density of 10 ml.

3. Pour off spent medium. Resuspend the cells in one tube to the 50-ml mark with Gamborg's B-5 medium supplemented with 20 g/L sucrose and 1 mg/L 2,4-D.

4. Resuspend the cells in the second tube with Gamborg's B-5 medium supplemented with 20 g/L sucrose, 1 mg/L 2,4-D, and 2.5 g/L yeast extract.

5. Resuspend the cells in the third tube with Gamborg's B-5 medium supplemented with 20 g/L sucrose, 1 mg/L 2,4-D, and 5.0 g/L yeast extract.

6. Resuspend the cells in the fourth tube with Gamborg's B-5 medium supplemented with 20 g/L sucrose, 1 mg/L 2,4-D, and 10.0 g/L yeast extract.

7. Transfer each treatment to its original 250-ml Erlenmeyer flask.

8. Incubate flasks at 25° C on a rotary shaker at 150 rpm. No light is required.

Harvesting and Extraction of Samples

1. Samples will be harvested immediately and after three days of culture.

2. For Day 0, immediately following inoculation of the cultures, pour the liquid contents of each flask into separate sterile 50-ml centrifuge tubes.

3. For each sample, allow cells to settle for 30 minutes. Then, pour off 30 ml of medium to a 100-ml graduate cylinder.

4. Add 30 ml of chloroform:methanol (50:50, v/v) solvent to the graduate cylinder. Transfer this mixture to a capped flask or other container for mixing.

5. Filter the mixture using Whatman 1PS phase separator paper. This allows the chloroform extract to pass through while retaining the water and methanol portion.

6. Collect the chloroform extract in a beaker and dry overnight at room temperature in a fume hood.

7. After each extraction, 30 ml of the appropriate fresh medium must be added to cells remaining in the centrifuge tubes and the suspensions returned to flasks for incubation.

8. Repeat the sampling and extraction procedure after three days of culture.

Thin-Layer Chromatography

1. Redissolve the extracts in 0.5 ml of chloroform.

2. Using a capillary pipet, spot 20 µl of each extract on to a 20 cm x 20 cm silica gel thin-layer chromatography plate.

3. These spots are started at a distance of about 2 cm from the lower edge of the plate and at least 1 cm from the side edges. The spacing of the spots for each sample should be about 10 mm. To apply 20 µl of sample requires applying one drop at a time and allowing the solvent to evaporate between applications.

4. Once all spots have dried, place the plate into a chromatography tank containing 100 ml of toluene:ethyl acetate (80:20, v/v).

5. Allow the solvent to ascend 15 cm past the starting point. Then remove and air dry.

6. Spray the plate with 2% paradimethylaminobenzaldehyde in concentrated $HCl:H_2O$ (50:50, v/v). Spraying must be done in a ventilated fume hood.

7. Heat the plate in an oven at 60° C until dry (about 10 minutes). Spots will be visible (Figure 23.1) in colors from gray to purple or blue.

8. Record the color and Rf (distance spot moved in mm/distance of solvent front in mm) values for each spot.

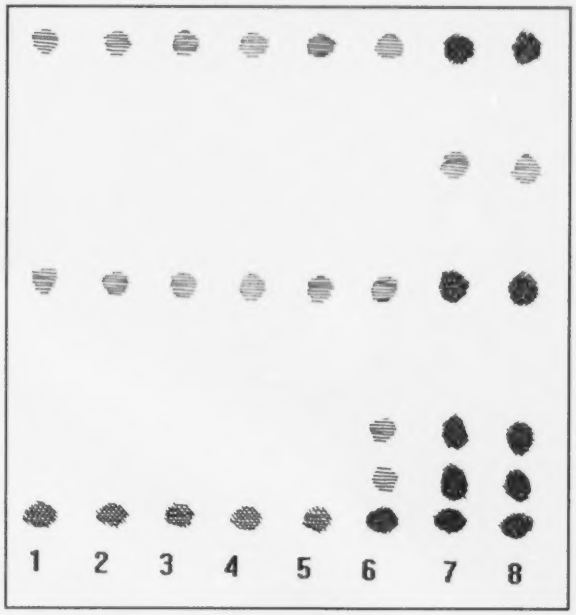

Figure 23.1. Thin-layer chromatogram of sweet potato suspension cultures. Lanes 1-4 are Day 0 of 0.0, 2.5, 5.0, and 10.0 g/L YE. Lanes 5-8 are Day 3 of 0.0, 2.5, 5.0, and 10.0 g/L YE.

Report 23
Induction of Phytoalexins in Sweet Potato Suspension Cultures

Name:

1. Record Rf values and color of spots here:

Spot Number	Rf Value	Color

2. Does yeast extract appear to induce phytoalexin compounds? Explain.

Optional

Design a study of your own using the investigation form provided in Appendix E. Following are some suggested questions:

1. Would other compounds, such as SDS (sodium dodecyl sulfate), be effective inducers of phytoalexins in sweet potato suspension cultures?
2. Would *Ceratocystis fimbriata* spores be effective at inducing phytoalexins using the system presented in this exercise?

24. Bioassay for Phytoalexins Induced in Sweet Potato Suspension Cultures

Objective

To gain experience in using a fungal bioassay system for detection of phytoalexins.

Background

In Exercise 23, an assumption is made that the spots seen on the thin-layer chromatogram are phytoalexin compounds. In this exercise, this assumption will be tested using spores of *Ceratocystis fimbriata*.

Materials

- 1 thin-layer silica gel plate, 20 cm x 20 cm
- 1 Sabouraud's dextrose agar plate seeded with *Ceratocystis fimbriata*, incubated for five days at 25° C
- 1 tube sterile distilled water, 10 ml
- 1 spatula
- 2 sterile applicator sticks
- 1 sterile 1-ml pipet
- 1 automatic pipetting device
- 10 uninoculated Sabouraud's dextrose agar plates
- incubator set at 25° C
- filter paper
- chromatography tank with 100 ml solvent (tuolene: ethyl acetate [80:20, v/v])
- capillary tubes

Procedure

Inoculation of SDA Plates

1. Use the sterile applicator to transfer spores from the plate containing *Ceratocystis fimbriata* mold to the tube of sterile water until you can see that the water is slightly turbid.

2. Inoculate the 10 fresh plates with Sabouraud's dextrose agar by transferring 0.1 ml of the spore suspension to each plate and using a sterile swab to spread the inoculum.

Isolation of Phytoalexins

1. For this exercise, use the yeast extract sample from Exercise 23 in which maximum induction of phytoalexins was obtained.

2. A second plate is now developed but not sprayed with detection spray. There should be from 15 to 20 lanes developed on this plate to obtain enough compounds for the bioassay.

3. Use a sprayed plate from Exercise 23 as a guide to scrape the silica gel for each spot from the second plate. Use the spatula to scrape all 15 to 20 spots for **each Rf value** into a separate petri dish. To do this, first scrape each spot into a piece of clean filter paper and then use the filter paper as a funnel to pour the silica gel into the center of the plate (Figure 24.1). In addition, scrape extra silica gel from below the origin to serve as a control.

4. Incubate plates at 25° C for five days and examine for evidence of inhibition (Figure 24.1).

5. Record the results on the report sheet and answer all the questions.

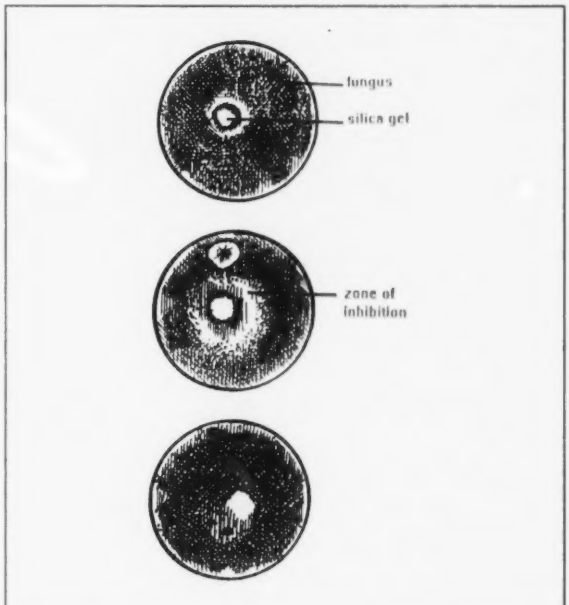

Figure 24.1. Sabouraud's dextrose agar plates with Ceratocystis fimbriata showing inhibitory zones.

Report 24
Bioassay for Phytoalexins Induced in Sweet Potato Suspension Cultures Name:

1. Which spots show inhibitory activity against *Ceratocystis fimbriata*?

2. If more than one group did the assay, were the results the same for each group? Explain.

Optional

Design a study of your own using the investigation form provided in Appendix E. Following are some suggested questions:

1. Would these compounds be inhibitory toward bacteria?
2. What effect would controlling the number of spores used as an inoculum have on the sensitivity of this assay?

25. Isolation of Oat Leaf Protoplasts

Objective

To gain experience using a protoplast isolation procedure.

Background

Protoplasts are plant cells from which the entire cell wall has been removed (Schaeffer, 1990). Plant protoplasts have been prepared from a number of sources, including leaves, petioles, roots, and flowers from greenhouse-grown plants (Eilers et al., 1988; Escandon et al., 1985; Oshiro & Steinhart, 1991), shoot cultures (Negrutiu & Mousseau, 1980; Creissen & Karp, 1985), and cell suspension cultures (Liu & Cantliffe, 1989; Sala et al., 1985). Regardless of the source, there are a number of variables that are crucial for success, including growth conditions of the donor plant, types and concentrations of enzymes used for isolation, and components of the protoplast culture medium (Evans & Bravo, 1983; Yasugi, 1989). Once these conditions are determined for each plant, however, the protoplasts may be used for fusion experiments useful for fundamental plant cell studies and for crop improvement (Morikawa & Yamada, 1992).

In this exercise, protoplasts will be isolated and their viabilities will be determined using leaf tissue of oat *(Avena sativa)* seedlings.

References

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Negrutiu, I. & Mousseau, J. (1980). Protoplast culture from *in vitro* grown plants of *Nicotiana sylvestris* Spegg. and Comes. *Zeitschrift fuer Pflanzenphysiologie, 100,* 373-376.

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Sala, C., Biasini, M.G., Morandi, C., Nielsen, E., Parisi, B. & Sala, F. (1985). Selection and nuclear DNA analysis of cell hybrids between *Daucus carota* and *Oryza sativa*. *Journal of Plant Physiology, 118,* 409-419.

Schaeffer, W.I. (1990). Terminology associated with cell, tissue and organ culture, molecular biology, and molecular genetics. *In Vitro Cellular and Developmental Biology, 26,* 97-101.

Yasugi, S. (1989). Isolation and culture of orchid protoplasts. In Y.P.S. Bajaj (Ed.), *Biotechnology in Agriculture and Forestry, Volume 8: Plant Protoplasts and Genetic Engineering,* 235-253. Berlin/Heidelberg: Springer-Verlag.

Materials

- oat *(Avena sativa)* seeds
- 1 6-inch pot with potting soil
- 1 large forceps, sterile
- 1 spatula, sterile
- 1 scalpel, sterile
- 1 300-ml beaker containing 250 ml 95% ethanol
- bleach solution, 10% (v/v), 300 ml (containing one drop of dish detergent
- 1 300-ml flask containing 250 ml sterile distilled water
- 10 ml enzyme solution (Gamborg's B-5 medium containing 30 g/L sucrose, 100 g/L mannitol, and 5 g/L cellulase). Adjust pH to 5.8. Sterilize using a 0.22 micrometer filter.
- 10 ml protoplast medium (This is the same composition as enzyme solution except that no enzyme is added.). Filter sterilize.

Additional Materials

- scissors
- sterile petri dishes (100 mm x 15 mm)
- plastic bags
- balance
- parafilm
- sterile 15-ml centrifuge tubes
- vacuum pump
- sterile 10-ml pipets
- 1 N HCl
- pH paper
- table top centrifuge
- Evans Blue reagent (1% Evans Blue stain in 13% mannitol)
- microscope with low and high power objectives
- microscope slides and coverslips
- incubator set at 25° C
- 0.22 micrometer filters

Procedure

Germination of Oat Seeds

1. Oat *(Avena sativa)* seeds are placed in a pot containing moistened potting soil. The seeds should be covered with about one-half inch of soil. Place the pot in a plastic bag to maintain humidity and incubate at 25° C with a 16-hour photoperiod (approximately 2,000 lux).

2. Once seedlings are four to six inches tall (about two weeks), use scissors to harvest 1 g of leaves.

Isolation of Protoplasts

1. Place the leaf material in a sterile petri dish and disinfect in 10% (v/v) bleach solution (containing one drop dish detergent/100 ml) for 10 minutes. Rinse three times with sterile distilled water.

2. Use sterile forceps and scalpel to cut the leaves lengthwise into 1-mm wide strips.

3. Place the strips into a sterile petri dish with 10 ml enzyme solution. The enzyme solution is Gamborg's B-5 medium containing 30 g/L sucrose, 100 g/L mannitol, and 5 g/L cellulase (Kaur-Sawhney & Galston, 1984). Adjust pH to 5.8. Sterilize using a 0.22 micrometer filter.

4. Seal the dish with parafilm and incubate in the dark at 25° C for two to three hours.

5. After incubation, use a sterile spatula to push undigested tissue to one end of the dish. Use a sterile pipet to transfer the protoplast suspension to a sterile 15-ml centrifuge tube.

6. Centrifuge at 2,000 rpm for five minutes and pour off supernatant.

7. Resuspend the protoplasts in 5 ml protoplast medium. This is the same composition as the enzyme solution except that no enzymes are used.

8. Centrifuge the mixture as before and pour off su-

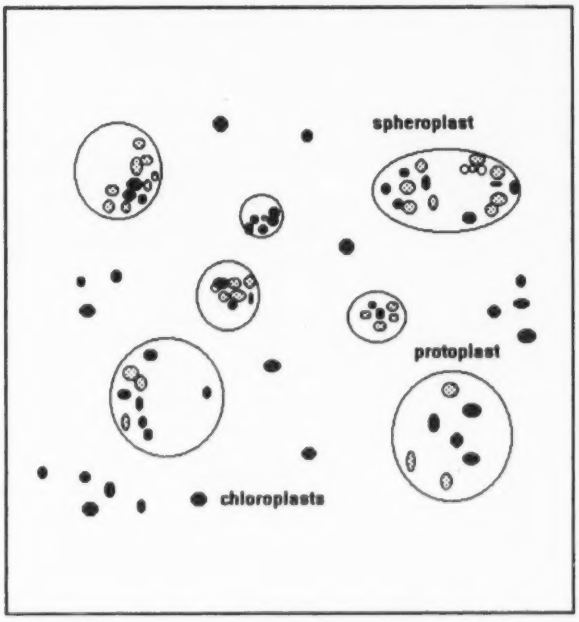

Figure 25.1. Oat leaf protoplasts as viewed under high power. Elongated cells indicate that the cell wall is not completely removed.

pernatant. Add 2 ml of protoplast medium to resuspend protoplasts.

9. Remove a drop of the suspension and examine microscopically using low power and high power objectives. Protoplasts should be spherical (Figure 25.1). Draw a representative sample in the table provided.

10. To test for viability, place one drop of protoplast suspension and one drop of Evans Blue reagent (1% Evans Blue stain in 13% mannitol) on a slide and examine using low power and high power. Intact protoplasts will exclude the dye.

11. Determine the percentage of viability using at least 500 protoplasts. Viability should not be below 50% to 60% (Ochatt & Power, 1992). Record results in the table provided.

Report 25
Isolation of Oat Leaf Protoplasts Name:

1. Observations of oat leaf protoplasts.

Microscopic Appearance	Percentage Viability

Optional

Design a study of your own using the investigative form provided in Appendix E. Following are some suggested questions:

1. What condition(s) might be altered to increase the number of protoplasts with cell walls completely removed?
2. If you did not have at least 50% viability, what condition(s) might be altered to increase viability?
3. Would these same conditions be effective for other systems, such as sweet potato or carrot cell cultures?

26. Isolation of Tobacco Leaf Protoplasts

Objective

To gain experience using a protoplast isolation procedure.

Background

Leaves of tobacco, *Nicotiana tabacum*, also provide protoplasts quite readily. In this exercise, the techniques used in the isolation of protoplasts from oat leaves will be applied to tobacco leaves.

Materials

(per student pair)

- 1 tobacco (*Nicotiana tabacum* cv. Wisconsin No. 38) plant
- 1 large forceps, sterile
- 1 spatula, sterile
- 1 scalpel, sterile
- 1 300-ml beaker containing 250 ml 95% ethanol
- bleach solution, 10% (v/v), 300 ml (containing one drop of dish detergent
- 1 300-ml flask containing 250 ml sterile distilled water
- 10 ml enzyme solution (Gamborg's B-5 medium containing 30 g/L sucrose, 100 g/L mannitol, and 5 g/L cellulase). Adjust pH to 5.8. Sterilize using a 0.22 micrometer filter.
- 10 ml protoplast medium (This is the same composition as enzyme solution except that no enzyme is added.). Filter sterilize.

Additional Materials

- scissors
- sterile petri dishes (100 mm x 15 mm)
- balance
- parafilm
- sterile 15-ml centrifuge tubes
- vacuum pump
- sterile 10-ml pipets
- 1 N HCl
- pH paper
- table top centrifuge
- Evans Blue reagent (1% Evans Blue stain in 13% mannitol)
- microscope with low and high power objectives
- microscope slides and coverslips
- incubator set at 25° C
- 0.22 micrometer filters

Procedure

1. Harvest 1 gram of leaf material. Place it in a sterile petri dish and disinfect in 10% (v/v) bleach solution (containing one drop dish detergent/100 ml) for 10 minutes. Rinse three times with sterile distilled water.

2. Use sterile forceps and scalpel to cut the leaves lengthwise into 1-mm wide strips.

3. Place the strips into a sterile petri dish with 10 ml enzyme solution. The enzyme solution is Gamborg's B-5 medium containing 30 g/L sucrose, 100 g/L mannitol, and 5 g/L cellulase. Adjust the pH to 5.8. Sterilize using a 0.22 micrometer filter.

4. Seal the dish with parafilm and incubate in the dark at 25° C overnight for 16 to 24 hours.

5. After incubation, use a sterile spatula to push undigested tissue to one end of the dish. Use a sterile pipet to transfer the protoplast suspension to a sterile 15-ml centrifuge tube.

6. Centrifuge at 2,000 rpm for five minutes and pour off supernatant.

7. Resuspend the protoplasts in 5 ml protoplast medium. This is the same composition as the enzyme solution except that no enzymes are used.

8. Centrifuge the mixture as before and pour off the

supernatant. Add 2 ml of protoplast medium to resuspend protoplasts.

9. Remove a drop of the suspension and examine microscopically using low power and high power objectives. Protoplasts should be spherical (Figure 25.1, Exercise 25). Draw a representative sample in the table provided.

10. To test for viability, place one drop of protoplast suspension and one drop of Evans Blue reagent (1% Evans Blue stain in 13% mannitol) on a slide and examine using low power and high power. Intact protoplasts will exclude the dye.

11. Determine the percentage viability using at least 500 protoplasts. Viability should not be below 50% to 60%. Record results in the table provided on the following page.

Report 26
Isolation of Tobacco Leaf Protoplasts

Name:

1. Observations of tobacco leaf protoplasts.

Microscopic Appearance	Percentage Viability

2. Does the percentage viability of protoplasts isolated for tobacco compare to the percentage viability of protoplasts for oats?

3. What is the main difference in procedure for isolation of protoplasts from tobacco compared to oats?

4. Were all the cells completely spherical or were some oblong? Explain.

5. Would additional enzymes, such as use of pectinase, increase the yield of protoplasts?

Optional

Design a study of your own using the investigative form provided in Appendix E. Following are some suggested questions:

1. Would using tobacco shoot cultures increase the percentage of viable cells?
2. Would using additional enzymes increase the yield of viable protoplasts?

APPENDIX A – INSTRUCTOR'S NOTES

Group Work

Students should work in pairs to make manipulations easier and to increase interaction during analysis of data. In addition, although it would be most appropriate for each pair of students to perform all replicates needed for an experiment, dividing the replicates among different student pairs and using class results reduces the amount of time and materials required.

Disinfection of Workspace, Instruments, and Plant Material

Experiments may be conducted on an open benchtop disinfected with 95% ethanol. Pour a generous amount of alcohol on the bench surface and use a paper towel to spread it over the surface. This is important to reduce the level of dust.

Instruments should be wrapped in aluminum foil and sterilized in an oven at 160° C for three hours. Allow to cool to room temperature and then place in a beaker containing 95% ethanol. Before use, just shake off excess alcohol. Do not flame the instruments due to the possibility of fire. Instruments are returned to the alcohol after use for disinfection. Do not depend on alcohol disinfection alone for reduction of bacterial and fungal contamination. Also, change the alcohol frequently since it may become contaminated with bacterial spores.

Twenty percent bleach solution is used for plant material. All disinfectant solutions should be prepared fresh each day.

Notes on Exercises

The exercises in *Unit I: The Process of Science* may be introduced during the "lecture" portion of the course. Prior to using computer programs for statistical analyses, allow the students to perform the calculations using the formulas presented in each case study. This aids their understanding of how each number is derived. It may be helpful to "walk through" the calculations for some students.

In succeeding units, the design and variety of exercises allow flexibility for instructor and student. It is recommended that students do at least one problem-solving application of each case study.

In exercises using fresh plant material or seeds (Exercises 8, 9, 10, 11, 14, 15, 18, 19, 25, and 26), best results will be obtained with the freshest material. Do not store sweet potato or carrot roots for long periods of time in the refrigerator. Purchase these items just prior to class. Do not use outdated pea or oat seeds.

For some activities (Exercises 12, 13, 23, and 24), callus established and subcultured for years may be used. It is not necessary to use freshly isolated callus.

For exercises using fresh plant material grown on agar plates (Exercises 8, 9, 10, and 11), make sure the explants do not roll over the surface of the plate and that the plate surface is not covered with droplets of moisture. Bacterial and fungal contaminants will spread under these conditions.

Student Projects

Student projects should be started early (e.g., during Weeks 3 to 5) to allow time for testing, reading and analysis of results, and preparation for presentation.

Hormone Stock Solutions

Use a 0.22 micrometer filter to sterilize the following solutions:

1. **1.0 mg/ml IAA.**
   Weigh 100 mg IAA and dissolve in a small amount of 1N NaOH. Bring the volume to 100 ml with distilled water.

2. **1.0 mg/ml Kinetin.**
   Weigh 100 mg Kinetin and dissolve in a small amount of 1N NaOH. Bring the volume to 100 ml with distilled water.

   In Exercises 20 and 21, Kinetin at 0.001 mg/L (1µg/L) is used. To prepare this dilution, transfer 1 ml of the above dilution to 9 ml of sterile distilled water and mix. This is 0.1 mg/L Kinetin. Then transfer 1 ml of this dilution to 9 ml of sterile distilled water to yield a 0.01 mg/L dilution. Finally, transfer 1 ml of this dilution to 9 ml of distilled water to yield 0.001 mg/L (1 µg/L) dilution.

3. **1.0 mg/ml NAA.**
   Weigh 100 mg NAA and dissolve in a small amount of 1N NaOH. Bring the volume to 100 ml with distilled water.

4. **1.0 mg/ml BA.**
   Weigh 100 mg BA and dissolve in a small amount of 1N NaOH. Bring the volume to 100 ml with distilled water.

5. **1.0 mg/L 2,4-D.**
   Weigh 100 mg 2,4-D and dissolve in a small amount of 95% ethanol. Bring the volume to 100 ml with distilled water.

Vitamins and Glycine Stock Solution

Weigh 50 mg nicotinic acid, 50 mg pyridoxine HCl, 40 mg thiamine HCl, and 200 mg glycine. Dissolve in a small volume of distilled water and then bring volume to 100 ml with distilled water.

Preparation of Tobacco Plants

To obtain tobacco plants, tobacco (*Nicotiana tabacum* cv. Wisconsin No. 38) shoots from tobacco shoot cultures are placed in a pot containing moistened potting soil, covered with a plastic bag to maintain humidity, and incubated at 25° C with a 16-hour photoperiod (approximately 2,000 lux) for three or four months for plant production. If preferred, shoot cultures may be used directly for experiments using tobacco leaves. The advantages to this are that disinfection of leaf material is not necessary, and there may be an increased success rate due to the elimination of this step.

Note: **1 N HCl** (for Exercises 20, 21) ... dilute 80 ml concentrated HCL to 1 liter distilled water.
1 N NaOH (for Exercises 20, 21) ... dissolve 41 g NaOH in 1 liter distilled water.

APPENDIX B – SUPPLIES DIRECTORY

American Type Culture Collection
12301 Parklawn Drive
Rockville, Maryland 20852-1776
Plant tissue cultures, bacterial cultures, fungal (Ceratocystis fimbriata) *cultures.*

Carolina Biological Supply Company
2700 York Road
Burlington, North Carolina 27215-3398
Plant tissue cultures, bacterial cultures, media, media supplements, chemicals, glass and plastic ware, filter units, other lab supplies.

Fisher Scientific
P.O. Box 3029
Malvern, Pennsylvania 19355
Chemicals, glass and plastic ware, filter units, Whatman #1 Phase separator paper.

Orchid Art
2433 Kew Avenue
Hewlett, New York 11557
Cattleya *and other orchids.*

Sigma Chemical Company
P.O. Box 14508
St. Louis, Missouri 63178-9916
Culture media, media supplements, chemicals, glass and plastic ware, filter units, other lab supplies.

Thomas Scientific
P.O. Box 99
Swedesboro, New Jersey 08085-6099
Chemicals, glass and plastic ware, filter units, eosinophil counting chamber.

Ward's
P.O. Box 92912
Rochester, New York 14692-9012
Plant tissue cultures, bacterial cultures, media, chemicals, glass and plastic ware, filter units, other lab supplies.

Harcourt Brace and Company
6277 Sea Harbor Drive
Orlando, Florida 32887
Joe Spreadsheet Statistical Program (limited stock available)

Addison-Wesley Publishing Company, Inc.
Educational Software Division
Jacob Way
Reading, Massachusetts 01867-9984
Minitab Statistical Software

Poly Software International
P.O. Box 526368
Salt Lake City, Utah 84152
PSI-Plot Statistical Software

APPENDIX C – CASE STUDY DISCUSSIONS

EXERCISE 2 – STUDENT *t* TEST

Analysis of Data

Before performing statistical analysis, the data should be examined to determine if there are any obvious or apparent trends. One obvious trend is that there is less variation in the 20% bleach data. That is, with 20% bleach, the number of uncontaminated explants ranges from four to five (4-5); whereas, with 95% ethanol, the numbers range from zero to five (0-5). At this point, it is not possible to tell if this variation is significant. That will be the function of the *F* test. Also, in examining the sample means, at first glance there does not appear to be an obvious difference. The number of uncontaminated explants with 20% bleach is 4.8 compared to 3.73 for 95% ethanol; however, on a percentage basis, this represents a greater than 21% increase. The *t* test will aid in determining if this is significant.

For this problem, a ***t* test** is appropriate for data analysis since it tests for differences between **two** independent samples. The formulas for performing this test are presented in Figure 2.2. The first step is to determine whether the variances of the two samples (s^2) differ significantly. To establish whether or not the variances of the two samples differ significantly, an *F* test is performed. The *F* value is **calculated** by dividing the larger by the smaller variance. In this case, $F = 2.78095/0.17143 = 16.22$. This value then is compared to the **critical value of *F***, obtained from tables presented in texts on biostatistical analysis, at the chosen level of significance. The 5% level of significance (alpha level) is commonly used as a criterion for rejection of the null hypothesis (Zar, 1996). In our case, we have 14 *df* in the numerator and 14 *df* in the denominator. Since this value, for a one-sided test, does not appear in the table, we use the critical value with the next lower *df*; i.e., 2.53. Since the calculated *F* value (16.22) is greater than the critical value of *F* (2.53), we reject the null hypothesis. That is, our variances are not equal. Thus, the t' test is the form of the ***t* test** that must be used if the variances of the two samples differ significantly. If the variances of the two samples do not differ significantly, then we would use the **Student *t* test**.

For the ***t'* test**, the calculated value of t is -2.4043. This is the same answer obtained by the Student *t* test; however, the *df* is calculated differently for the *t'* test (Figure 2.2). We now have to compare this **calculated value of *t*** to the **critical value of *t*** at the 0.05 level of signficance with 16 *df*. Again, the 5% level of significance (alpha level) is commonly used as a criterion for rejection of the null hypothesis (Zar, 1996). As for the critical value of *F*, this critical value of *t* may be obtained from a table found in texts of statistical analysis; however, the critical value of *t* depends upon your alternative hypothesis. Since there was reason to suspect bleach was more effective than alcohol for disinfection, this is a directional or one-sided test (Schmidt, 1979). Thus, the critical value of *t* is -1.746. Since the calculated *t* value falls in the critical region (i.e., since the calculated *t* value is larger than the critical value of *t*), we reject our null hypothesis. That is, the number of uncontaminated explants obtained with bleach treatment is significantly more than that obtained with ethanol.

Discussion

Another concept in the interpretation of statistical tests is that of practical significance. For the sample study, a statistically significant difference between the two population means was found; however, is this of practical value? In looking at actual numbers, disinfection with bleach allowed 72 of 75 explants to survive. On the other

hand, disinfection with alcohol did allow 56 of the 75 explants to survive. For some research systems, this difference is not important. In a classroom situation, however, this is of practical value. The higher rate of survival at the disinfection step would yield more data for analysis at the end of each experiment. Thus, for greater classroom success, bleach disinfection is preferred.

The decisions for this statistical analysis can be difficult. Depending upon the research objective, other levels of significance may be chosen. Also statistical tests are sensitive to sample size. This is why it is best to consult a statistician for advice in the design of experiments.

References

Schmidt, M.J. (1979). *Understanding and Using Statistics: Basic Concepts* (2nd ed.). Lexington, MA: D.C. Heath and Company.

Zar, J.H. (1996). *Biostatistical Analysis* (3rd ed.). Upper Saddle River, NJ: Prentice-Hall, Inc.

EXERCISE 3 – ONE-WAY ANOVA

Analysis of Data

As with Exercise 2, before performing statistical analysis, the data should be examined to determine if there are any obvious or apparent trends. In examining the results in Table 3.1, the average number of embryos for the 0.0 and 0.5 g/L casein hydrolysate appear to be close, but the number for the 1.0 and 2.0 g/L casein hydrolysate both appear to be different from each other and from the first two levels. A **one-way analysis of variance** (ANOVA) will aid in determining if these differences are significant.

In this example, the single factor analysis of variance is appropriate for data analysis since there are more than two samples. The purpose of performing this test is to aid in determining if the observed difference in sample means is due to the effect of casein hydrolysate or embryogenesis or if it is due simply to sampling variability. Analysis of the sample results from Table 3.1 is presented below.

Source of Variation	*SS*	*df*	*MS*	*F*	F_{crit}
Between Groups	91585.48	3	30528.49	33.73842	2.92
Within Groups	33574.90	36	904.8583		
Total	124160.4	39	3183.599		

The $F_{critical}$ **value** for these sample results at the 0.05 alpha level for $df = 3,30$ is 2.92 (since $df = 3,36$ is not on the table, the F value for the next lower df is used). If a statistical program such as the *Joe Spreadsheet Statistical Program* (Goldstein Software, Inc., Harcourt Brace & Company, Orlando, FL), *Minitab Statistical Software* (Minitab, Inc., Addison-Wesley Publishing Company, Inc., Reading, MA), or *PSI-Plot* (Poly Software International, Salt Lake City, UT) is used, it will compute this value exactly (2.866266) and may compute a *p-value*. In

this example, the observed, or calculated F value is greater than $F_{critical}$. Thus, we reject the null hypothesis. We conclude that casein hydrolysate did have an effect on the number of embryos formed.

Discussion

These results agree with those obtained by Masuda et al. (1981). Masuda et al. (1981) determined that addition of casein hydrolysate increased embryogenesis by up to 160%. They used Murashige and Skoog medium modified by the concentrations of NH_4NO_3 and KNO_3 to 10.3 mM and 24.7 mM, respectively. Gamborg's B-5 medium, which was used in this study, contains $(NH_4)_2SO_4$ and KNO_3 at 1.01 mM and 24.7 mM, respectively. Thus, an increase in embryogenesis might be expected since potassium nitrate as a sole source of nitrogen shows very low frequency of embryogenesis (Masuda et al., 1981), and organic nitrogen may partially replace the ammonium as a supplement (Wetherell & Dougall, 1976).

References

Masuda, K., Kikuta, Y. & Okazawa, Y. (1981). A revision of the medium for somatic embryogenesis in carrot suspension culture. *Journal of the Faculty of Agriculture., Hokkaido University, 60,* 183-192.

Wetherell, D.F. & Dougall, D.K. (1976). Sources of nitrogen supporting growth and embryogenesis in cultured wild carrot tissue. *Physiologie Plantarum, 37,* 97-103.

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EXERCISE 4 – TWO-WAY ANOVA

Analysis of Data

For this experiment, the **dependent variable** under consideration is the average number of shoots forming per explant. This is what we are trying to influence by varying the concentrations of benzylaminopurine (BA) and naphthaleneacetic acid (NAA). The two **independent variables** (factors) are the BA and NAA concentrations. In examining the data for this experiment (Table 4.1), one apparent trend is that the number of shoots forming on media containing BA is more than four times the number forming on media without BA. It would appear that BA does have an influence on the number of shoots forming. Since there are two levels of BA and three levels of NAA, a **two-way analysis of variance** (ANOVA) will aid in determining if this difference is significant.

Analysis of sample data from Table 4.1 is presented below:

Source of Variation	*SS*	*df*	*MS*	*F*	F_{crit}
Column	1180.083	1	1180.083	387.97	4.08
Row	26.00	2	13.00	4.27	3.23
Interaction	22.167	2	11.083	3.64	3.23
Within Groups	127.750	42	3.042		
Total	1356.00	47	28.042		

Discussion

For a two-way ANOVA, the **type of factor** (**fixed** or **random**) that is being considered must be taken into account. In this study, the levels used for each factor were fixed based upon previous work. Griga et al. (1984) found that 20 μM (4.5 mg/L) BA combined with 0.1 μM (0.02 mg/L) NAA gave the highest shoot proliferation for *Pisum sativum* L. Bohatyr. Initial trials at the 4.5 mg/L BA concentration for the peas used in this study resulted in a high percentage of death. Decreasing the concentration of BA to 0.45 mg/L resolved this problem. Since these levels were fixed based upon previous research, generalizations about observed effects are made only to the levels used in this experiment (Schmidt, 1979). A factor is considered random if the levels chosen for the experiment are picked randomly from a very large population of potential levels. Generalizations then may be made to levels other than those used in the study (Schmidt, 1979).

A 5% level of significance (alpha level) was used as a criterion for rejection of the null hypothesis. The critical F value for the column results for $df = 1,40$ (since df = 1,42 is not on the table, the F value for the next lower df is used) is 4.08. Similarly, the critical F value for the row and interaction results for $df = 2,40$ (since $df = 2,42$ is not on the table) is 3.23. Since all calculated F-values are greater than the critical value of F, we reject the null hypotheses. That is, the 0.45 mg/L BA concentration did influence average number of pea shoot tips produced. Also, NAA influenced average number of pea shoot tips produced, and there were significant interactions between the two hormones that influenced average number of shoot tips produced.

References

Griga, M., Tejklova, E. & Novak, F.J. (1984). Hormonal regulation of growth of pea (*Pisum sativum* L.) shoot apices in *in-vitro* culture. *Rostlinna Vyroba, 30,* 523-530.

Schmidt, M.J. (1979). *Understanding and Using Statistics: Basic Concepts* (2nd ed.). Lexington, MA: D.C. Heath and Company.

EXERCISE 5 – THE TUKEY TEST

Analysis of Data

Since NAA was determined to have a significant influence on number of shoots for peas (Exercise 4), and since there were more than two levels of NAA, then a multiple comparison procedure, such as the **Tukey test**, should be used to decide which pairs of means are significantly different. To do this test, a ***q* value** is calculated and compared to the **critical value of *q*** ($q_{alpha,v,k}$), where v = the within group df and k = the number of means being tested (Zar, 1996). Since $q_{0.05,42,3}$ does not appear in the table, the critical value with the next lower df is used. If the observed, or calculated, q value is equal to or greater than $q_{critical}$, then the null hypothesis is rejected. The results of this analysis are presented below:

Comparison (B vs. A)	Difference ($\bar{X}_B - \bar{X}_A$)	*SE*	*q*	$q_{0.05,40,3}$	Conclusion
2 vs. 1	1.75	0.436	4.0138	3.442	reject
2 vs. 3	1.25	0.436	2.8669	3.442	accept
3 vs. 1	0.5	0.436	1.1468	3.442	accept

Tukey multiple comparison test for NAA concentration. Alpha = 0.05

Discussion

The Tukey test revealed that the average number of pea shoots in the 0.02 mg/L NAA group is significantly higher than that of the 0.0 mg/L group, but not the 0.2 mg/L group; however, the 0.2 mg/L group is not significantly different from the 0.0 mg/L group. Also, since the average number of pea shoots in the 0.45 mg/L BA (Exercise 4) and 0.02 mg/L NAA cell-interaction is higher than all other cells, we concluded that this cell-interaction is optimum for shoot tip production.

These sample results did not compare to those of Griga et al. (1984). They found that 20 μM (approximately 4.5 mg/L) BA combined with 0.1 μM (approximately 0.02 mg/L) NAA gave the highest rate of shoot proliferation. That is, the effective BA concentration in our study was 1/10 the concentration used in their study. What can explain the difference between these studies? One possibility is error in hormone concentration. This would be a **systematic error**. Statistical tests aid in the evaluation of random error, not systematic error. Systematic error is difficult to detect; however, repeating the experiment with different media and reagents would aid in detection of systematic error. A repeat of the experiment using fresh stock solutions of hormones and media yielded similar results.

Another possibility might be differences due to **variety** or **genotype**. Treatment differences in shoot regeneration response for three genotypes were reported for *Populus deltoides* (Coleman & Ernst, 1989). The pea variety "Little Marvel" was used in our study. Griga et al. (1984) used the pea variety "Bohatyr." Further testing is needed to provide support for this hypothesis for these varieties of peas.

References

Coleman, G.D. & Ernst, S.G. (1989). *In vitro* shoot regeneration of *Populus deltoides:* Effect of cytokinin and genotype. *Plant Cell Reports, 8,* 459-462.

Griga, M., Tejklova, E. & Novak, F.J. (1984). Hormonal regulation of growth of pea (*Pisum sativum* L.) shoot apices in *in-vitro* culture. *Rostlinna Vyroba, 30,* 523-530.

Zar, J.H. (1996). *Biostatistical Analysis* (3rd ed.). Upper Saddle River, NJ: Prentice-Hall, Inc.

APPENDIX D – STATISTICAL TABLES

Table D. 1a. Critical values of the *F*-distribution calculated in the Student *t* test, one-tailed, and for the ANOVA test. Alpha = 0.05.

df den.	*df* num.								
	1	2	3	4	5	6	7	8	9
1	161	200	216	225	230	234	237	239	241
2	18.5	19.0	19.2	19.2	19.3	19.3	19.3	19.4	19.4
3	10.1	9.55	9.28	9.12	9.01	8.94	8.89	8.85	8.81
4	7.71	6.94	6.59	6.39	6.26	6.16	6.09	6.04	6.00
5	6.61	5.79	5.41	5.19	5.05	4.95	4.88	4.82	4.77
6	5.99	5.14	4.76	4.53	4.39	4.28	4.21	4.15	4.10
7	5.59	4.74	4.35	4.12	3.97	3.87	3.79	3.73	3.68
8	5.32	4.46	4.07	3.84	3.69	3.58	3.50	3.44	3.39
9	5.12	4.26	3.86	3.63	3.48	3.37	3.29	3.23	3.18
10	4.96	4.10	3.71	3.48	3.33	3.22	3.14	3.07	3.02
11	4.84	3.98	3.59	3.36	3.20	3.09	3.01	2.95	2.90
12	4.75	3.89	3.49	3.26	3.11	3.00	2.91	2.85	2.80
13	4.67	3.81	3.41	3.18	3.03	2.92	2.83	2.77	2.71
14	4.60	3.74	3.34	3.11	2.96	2.85	2.76	2.70	2.65
15	4.54	3.68	3.29	3.06	2.90	2.79	2.71	2.64	2.59
16	4.49	3.63	3.24	3.01	2.85	2.74	2.66	2.59	2.54
17	4.45	3.59	3.20	2.96	2.81	2.70	2.61	2.55	2.49
18	4.41	3.55	3.16	2.93	2.77	2.66	2.58	2.51	2.46
19	4.38	3.52	3.13	2.90	2.74	2.63	2.54	2.48	2.42
20	4.35	3.49	3.10	2.87	2.71	2.60	2.51	2.45	2.39
21	4.32	3.47	3.07	2.84	2.68	2.57	2.49	2.42	2.37
22	4.30	3.44	3.05	2.82	2.66	2.55	2.46	2.40	2.34
23	4.28	3.42	3.03	2.80	2.64	2.53	2.44	2.37	2.32
24	4.26	3.40	3.01	2.78	2.62	2.51	2.42	2.36	2.30
25	4.24	3.39	2.99	2.76	2.60	2.49	2.40	2.34	2.28
26	4.23	3.37	2.98	2.74	2.59	2.47	2.39	2.32	2.27
27	4.21	3.35	2.96	2.73	2.57	2.46	2.37	2.31	2.25
28	4.20	3.34	2.95	2.71	2.56	2.45	2.36	2.29	2.24
29	4.18	3.33	2.93	2.70	2.55	2.43	2.35	2.28	2.22
30	4.17	3.32	2.92	2.69	2.53	2.42	2.33	2.27	2.21
40	4.08	3.23	2.84	2.61	2.45	2.34	2.25	2.18	2.12
60	4.00	3.15	2.76	2.53	2.37	2.25	2.17	2.10	2.04
120	3.92	3.07	2.68	2.45	2.29	2.17	2.09	2.02	1.96
∞	3.84	3.00	2.60	2.37	2.21	2.10	2.01	1.94	1.88

Source: Reprinted by permission from Table B.4, Zar, J.H. (1996). *Biostatistical Analysis* (3rd ed.). Upper Saddle River, NJ: Prentice-Hall, Inc.

Table D. 1b. Critical values of the *F*-distribution calculated in the Student *t* test, one-tailed, and for the ANOVA test. Alpha = 0.05.

df den.	*df* num.									
	10	12	15	20	24	30	40	60	120	∞
1	242	244	246	248	249	250	251	252	253	254
2	19.4	19.4	19.4	19.4	19.5	19.5	19.5	19.5	19.5	19.5
3	8.79	8.74	8.70	8.66	8.64	8.62	8.59	8.57	8.55	8.53
4	5.96	5.91	5.86	5.80	5.77	5.75	5.72	5.69	5.66	5.63
5	4.74	4.68	4.62	4.56	4.53	4.50	4.46	4.43	4.40	4.36
6	4.06	4.00	3.94	3.87	3.84	3.81	3.77	3.74	3.70	3.67
7	3.64	3.57	3.51	3.44	3.41	3.38	3.34	3.30	3.27	3.23
8	3.35	3.28	3.22	3.15	3.12	3.08	3.04	3.01	2.97	2.93
9	3.14	3.07	3.01	2.94	2.90	2.86	2.83	2.79	2.75	2.71
10	2.98	2.91	2.85	2.77	2.74	2.70	2.66	2.62	2.58	2.54
11	2.85	2.79	2.72	2.65	2.61	2.57	2.53	2.49	2.45	2.40
12	2.75	2.69	2.62	2.54	2.51	2.47	2.43	2.38	2.34	2.30
13	2.67	2.60	2.53	2.46	2.42	2.38	2.34	2.30	2.25	2.21
14	2.60	2.53	2.46	2.39	2.35	2.31	2.27	2.22	2.18	2.13
15	2.54	2.48	2.40	2.33	2.29	2.25	2.20	2.16	2.11	2.07
16	2.49	2.42	2.35	2.28	2.24	2.19	2.15	2.11	2.06	2.01
17	2.45	2.38	2.31	2.23	2.19	2.15	2.10	2.06	2.01	1.96
18	2.41	2.34	2.27	2.19	2.15	2.11	2.06	2.02	1.97	1.92
19	2.38	2.31	2.23	2.16	2.11	2.07	2.03	1.98	1.93	1.88
20	2.35	2.28	2.20	2.12	2.08	2.04	1.99	1.95	1.90	1.84
21	2.32	2.25	2.18	2.10	2.05	2.01	1.96	1.92	1.87	1.81
22	2.30	2.23	2.15	2.07	2.03	1.98	1.94	1.89	1.84	1.78
23	2.27	2.20	2.13	2.05	2.01	1.96	1.91	1.86	1.81	1.76
24	2.25	2.18	2.11	2.03	1.98	1.94	1.89	1.84	1.79	1.73
25	2.24	2.16	2.09	2.01	1.96	1.92	1.87	1.82	1.77	1.71
26	2.22	2.15	2.07	1.99	1.95	1.90	1.85	1.80	1.75	1.69
27	2.20	2.13	2.06	1.97	1.93	1.88	1.84	1.79	1.73	1.67
28	2.19	2.12	2.04	1.96	1.91	1.87	1.82	1.77	1.71	1.65
29	2.18	2.10	2.03	1.94	1.90	1.85	1.81	1.75	1.70	1.64
30	2.16	2.09	2.01	1.93	1.89	1.84	1.79	1.74	1.68	1.62
40	2.08	2.00	1.92	1.84	1.79	1.74	1.69	1.64	1.58	1.51
60	1.99	1.92	1.84	1.75	1.70	1.65	1.59	1.53	1.47	1.39
120	1.91	1.83	1.75	1.66	1.61	1.55	1.50	1.43	1.35	1.25
∞	1.83	1.75	1.67	1.57	1.52	1.46	1.39	1.32	1.22	1.00

Source: Reprinted with permission from Table B.4, Zar, J.H. (1996). *Biostatistical Analysis* (3rd ed.). Upper Saddle River, NJ: Prentice-Hall, Inc.

Table D. 1c. Critical values of the *F*-distribution calculated in the Student *t* test, two-tailed. Alpha = 0.05.

df den.	*df* num.								
	1	2	3	4	5	6	7	8	9
1	648	800	864	900	922	937	948	957	963
2	38.5	39.0	39.2	39.2	39.3	39.3	39.4	39.4	39.4
3	17.4	16.0	15.4	15.1	14.9	14.7	14.6	14.5	14.5
4	12.2	10.6	9.98	9.60	9.36	9.20	9.07	8.98	8.90
5	10.0	8.43	7.76	7.39	7.15	6.98	6.85	6.76	6.68
6	8.81	7.26	6.60	6.23	5.99	5.82	5.70	5.60	5.52
7	8.07	6.54	5.89	5.52	5.29	5.12	4.99	4.90	4.82
8	7.57	6.06	5.42	5.05	4.82	4.65	4.53	4.43	4.36
9	7.21	5.71	5.08	4.72	4.48	4.32	4.20	4.10	4.03
10	6.94	5.46	4.83	4.47	4.24	4.07	3.95	3.85	3.78
11	6.72	5.26	4.63	4.28	4.04	3.88	3.76	3.66	3.59
12	6.55	5.10	4.47	4.12	3.89	3.73	3.61	3.51	3.44
13	6.41	4.97	4.35	4.00	3.77	3.60	3.48	3.39	3.31
14	6.30	4.86	4.24	3.89	3.66	3.50	3.38	3.29	3.21
15	6.20	4.77	4.15	3.80	3.58	3.41	3.29	3.20	3.12
16	6.12	4.69	4.08	3.73	3.50	3.34	3.22	3.12	3.05
17	6.04	4.62	4.01	3.66	3.44	3.28	3.16	3.06	2.98
18	5.98	4.56	3.95	3.61	3.38	3.22	3.10	3.01	2.93
19	5.92	4.51	3.90	3.56	3.33	3.17	3.05	2.96	2.88
20	5.87	4.46	3.86	3.51	3.29	3.13	3.01	2.91	2.84
21	5.83	4.42	3.82	3.48	3.25	3.09	2.97	2.87	2.80
22	5.79	4.38	3.78	3.44	3.22	3.05	2.93	2.84	2.76
23	5.75	4.35	3.75	3.41	3.18	3.02	2.90	2.81	2.73
24	5.72	4.32	3.72	3.38	3.15	2.99	2.87	2.78	2.70
25	5.69	4.29	3.69	3.35	3.13	2.97	2.85	2.75	2.68
26	5.66	4.27	3.67	3.33	3.10	2.94	2.82	2.73	2.65
27	5.63	4.24	3.65	3.31	3.08	2.92	2.80	2.71	2.63
28	5.61	4.22	3.63	3.29	3.06	2.90	2.78	2.69	2.61
29	5.59	4.20	3.61	3.27	3.04	2.88	2.76	2.67	2.59
30	5.57	4.18	3.59	3.25	3.03	2.87	2.75	2.65	2.57
40	5.42	4.05	3.46	3.13	2.90	2.74	2.62	2.53	2.45
60	5.29	3.93	3.34	3.01	2.79	2.63	2.51	2.41	2.33
120	5.15	3.80	3.23	2.89	2.67	2.52	2.39	2.30	2.22
∞	5.02	3.69	3.12	2.79	2.57	2.41	2.29	2.19	2.11

Source: Reprinted with permission from Table B.4, Zar, J.H. (1996). *Biostatistical Analysis* (3rd ed.). Upper Saddle River, NJ: Prentice-Hall, Inc.

Table D. 1d. Critical values of the *F*-distribution calculated in the Student *t* test, two-tailed. Alpha = 0.05.

df den.	*df* num.									
	10	12	15	20	24	30	40	60	120	∞
1	969	977	985	993	997	1000	1010	1010	1010	1020
2	39.4	39.4	39.4	39.4	39.5	39.5	39.5	39.5	39.5	39.5
3	14.4	14.3	14.3	14.2	14.1	14.1	14.0	14.0	13.9	13.9
4	8.84	8.75	8.66	8.56	8.51	8.46	8.41	8.36	8.31	8.26
5	6.62	6.52	6.43	6.33	6.28	6.23	6.18	6.12	6.07	6.02
6	5.46	5.37	5.27	5.17	5.12	5.07	5.01	4.96	4.90	4.85
7	4.76	4.67	4.57	4.47	4.42	4.36	4.31	4.25	4.20	4.14
8	4.30	4.20	4.10	4.00	3.95	3.89	3.84	3.78	3.73	3.67
9	3.96	3.87	3.77	3.67	3.61	3.56	3.51	3.45	3.39	3.33
10	3.72	3.62	3.52	3.42	3.37	3.31	3.26	3.20	3.14	3.08
11	3.53	3.43	3.33	3.23	3.17	3.12	3.06	3.00	2.94	2.88
12	3.37	3.28	3.18	3.07	3.02	2.96	2.91	2.85	2.79	2.72
13	3.25	3.15	3.05	2.95	2.89	2.84	2.78	2.72	2.66	2.60
14	3.15	3.05	2.95	2.84	2.79	2.73	2.67	2.61	2.55	2.49
15	3.06	2.96	2.86	2.76	2.70	2.64	2.59	2.52	2.46	2.40
16	2.99	2.89	2.79	2.68	2.63	2.57	2.51	2.45	2.38	2.32
17	2.92	2.82	2.72	2.62	2.56	2.50	2.44	2.38	2.32	2.25
18	2.87	2.77	2.67	2.56	2.50	2.44	2.38	2.32	2.26	2.19
19	2.82	2.72	2.62	2.51	2.45	2.39	2.33	2.27	2.20	2.13
20	2.77	2.68	2.57	2.46	2.41	2.35	2.29	2.22	2.16	2.09
21	2.73	2.64	2.53	2.42	2.37	2.31	2.25	2.18	2.11	2.04
22	2.70	2.60	2.50	2.39	2.33	2.27	2.21	2.14	2.08	2.00
23	2.67	2.57	2.47	2.36	2.30	2.24	2.18	2.11	2.04	1.97
24	2.64	2.54	2.44	2.33	2,27	2.21	2.15	2.08	2.01	1.94
25	2.61	2.51	2.41	2.30	2.24	2.18	2.12	2.05	1.98	1.91
26	2.59	2.49	2.39	2.28	2.22	2.16	2.09	2.03	1.95	1.88
27	2.57	2.47	2.36	2.25	2.19	2.13	2.07	2.00	1.93	1.85
28	2.55	2.45	2.34	2.23	2.17	2.11	2.05	1.98	1.91	1.83
29	2.53	2.43	2.32	2.21	2.15	2.09	2.03	1.96	1.89	1.81
30	2.51	2.41	2.31	2.20	2.14	2.07	2.01	1.94	1.87	1.79
40	2.39	2.29	2.18	2.07	2.01	1.94	1.88	1.80	1.72	1.64
60	2.27	2.17	2.06	1.94	1.88	1.82	1.74	1.67	1.58	1.48
120	2.16	2.05	1.94	1.82	1.76	1.69	1.61	1.53	1.43	1.31
∞	2.05	1.94	1.83	1.71	1.64	1.57	1.48	1.39	1.27	1.00

Source: Reprinted with permission from Table B.4, Zar, J.H. (1996). *Biostatistical Analysis* (3rd ed.). Upper Saddle River, NJ: Prentice-Hall, Inc.

Table D.2. Critical values of the *t* distribution. Alpha = 0.05.

(n) degrees of freedom	one-tailed test	two-tailed test
1	6.314	12.706
2	2.920	4.303
3	2.353	3.182
4	2.132	2.776
5	2.015	2.571
6	1.943	2.447
7	1.895	2.365
8	1.860	2.306
9	1.833	2.262
10	1.812	2.228
11	1.796	2.201
12	1.782	2.179
13	1.771	2.160
14	1.761	2.145
15	1.753	2.131
16	1.746	2.120
17	1.740	2.110
18	1.734	2.101
19	1.729	2.093
20	1.725	2.086
21	1.721	2.080
22	1.717	2.074
23	1.714	2.069
24	1.711	2.064
25	1.708	2.060
26	1.706	2.056
27	1.703	2.052
28	1.701	2.048
29	1.699	2.045
30	1.697	2.042
40	1.684	2.021
60	1.671	2.000
120	1.658	1.980
∞	1.645	1.960

Source: Repinted with permission of Houghton Mifflin Company from Table B, Schmidt, M.J. (1979). *Understanding and Using Statistics, Basic Concepts* (2nd ed.). Lexington, MA: D.C. Heath and Company

Table D.3a. Critical values of the *q* distribution. Alpha = 0.05.

v	p = 2	3	4	5	6	7	8	9	10
1	17.97	26.98	32.82	37.08	40.41	43.12	45.40	47.36	49.07
2	6.085	8.331	9.798	10.88	11.74	12.44	13.03	13.54	13.99
3	4.501	5.910	6.825	7.502	8.037	8.478	8.853	9.177	9.462
4	3.927	5.040	5.757	6.287	6.707	7.053	7.347	7.602	7.826
5	3.635	4.602	5.218	5.673	6.033	6.330	6.582	6.802	6.995
6	3.461	4.339	4.896	5.305	5.628	5.895	6.122	6.319	6.493
7	3.344	4.165	4.681	5.060	5.359	5.606	5.815	5.998	6.158
8	3.261	4.041	4.529	4.886	5.167	5.399	5.597	5.767	5.918
9	3.199	3.949	4.415	4.756	5.024	5.244	5.432	5.595	5.739
10	3.151	3.877	4.327	4.654	4.912	5.124	5.305	5.461	5.599
11	3.113	3.820	4.256	4.574	4.823	5.028	5.202	5.353	5.487
12	3.082	3.773	4.199	4.508	4.751	4.950	5.119	5.265	5.395
13	3.055	3.735	4.151	4.453	4.690	4.885	5.049	5.192	5.318
14	3.033	3.702	4.111	4.407	4.639	4.829	4.990	5.131	5.254
15	3.014	3.674	4.076	4.367	4.595	4.782	4.940	5.077	5.198
16	2.998	3.649	4.046	4.333	4.557	4.741	4.897	5.031	5.150
17	2.984	3.628	4.020	4.303	4.524	4.705	4.858	4.991	5.108
18	2.971	3.609	3.997	4.277	4.495	4.673	4.824	4.956	5.071
19	2.960	3.593	3.977	4.253	4.469	4.645	4.794	4.924	5.038
20	2.950	3.578	3.958	4.232	4.445	4.620	4.768	4.896	5.008
24	2.919	3.532	3.901	4.166	4.373	4.541	4.684	4.807	4.915
30	2.888	3.486	3.845	4.102	4.302	4.464	4.602	4.720	4.824
40	2.858	3.442	3.791	4.039	4.232	4.389	4.521	4.635	4.735
60	2.829	3.399	3.737	3.977	4.163	4.314	4.441	4.550	4.646
120	2.800	3.356	3.685	3.917	4.096	4.241	4.363	4.468	4.560
∞	2.772	3.314	3.633	3.858	4.030	4.170	4.286	4.387	4.474

Source: Reprinted with permission from Table B.5, Zar, J.H. (1996). *Biostatistical Analysis* (3rd ed.). Upper Saddle River, NJ: Prentice-Hall, Inc.

Table D.3b. Critical values of the *q* distribution. Alpha = 0.05.

v	p = 11	12	13	14	15	16	17	18	19
1	50.59	51.96	53.20	54.33	55.36	56.32	57.22	58.04	58.83
2	14.39	14.75	15.08	15.38	15.65	15.91	16.14	16.37	17.57
3	9.717	9.946	10.15	10.35	10.53	10.69	10.84	10.98	11.11
4	8.027	8.208	8.373	8.525	8.664	8.794	8.914	9.028	9.134
5	7.168	7.324	7.466	7.596	7.717	7.828	7.932	8.030	8.122
6	6.649	6.789	6.917	7.034	7.143	7.244	7.338	7.426	7.508
7	6.302	6.431	6.550	6.658	6.759	6.852	6.939	7.020	7.097
8	6.054	6.175	6.287	6.389	6.483	6.571	6.653	6.729	6.802
9	5.867	5.983	6.089	6.186	6.276	6.359	6.437	6.510	6.579
10	5.722	5.833	5.935	6.028	6.114	6.194	6.269	6.339	6.405
11	5.605	5.713	5.811	5.901	5.984	6.062	6.134	6.202	6.265
12	5.511	5.615	5.710	5.798	5.878	5.953	6.023	6.089	6.151
13	5.431	5.533	5.625	5.711	5.789	5.862	5.931	5.995	6.055
14	5.364	5.463	5.554	5.637	5.714	5.586	5.852	5.915	5.974
15	5.306	5.404	5.493	5.574	5.649	5.720	5.785	5.846	5.904
16	5.256	5.352	5.439	5.520	5.593	5.662	5.727	5.786	5.843
17	5.212	5.307	5.392	5.471	5.544	5.612	5.675	5.734	5.790
18	5.174	5.267	5.352	5.429	5.501	5.568	5.630	5.688	5.743
19	5.140	5.231	5.315	5.391	5.462	5.528	5.589	5.647	5.701
20	5.108	5.199	5.282	5.357	5.427	5.493	5.553	5.610	5.663
24	5.012	5.099	5.179	5.251	5.319	5.381	5.439	5.494	5.545
30	4.917	5.001	5.077	5.147	5.211	5.271	5.327	5.379	5.429
40	4.824	4.904	4.977	5.044	5.106	5.163	5.216	5.266	5.313
60	4.732	4.808	4.878	4.942	5.001	5.056	5.107	5.154	5.199
120	4.641	4.714	4.781	4.842	4.898	4.950	4.998	5.044	5.086
∞	4.552	4.622	4.685	4.743	4.796	4.845	4.891	4.934	4.974

Source: Reprinted with permission from Table B.5, Zar, J.H. (1996). *Biostatistical Analysis* (3rd ed.). Upper Saddle River, NJ: Prentice-Hall, Inc.

Table D.3c. Critical values of the *q* distribution. Alpha 0.05.

v	p = 20	22	24	26	28	30	32	34	36
1	59.56	60.91	62.12	63.22	64.23	65.15	66.01	66.81	67.56
2	16.77	17.13	17.45	17.75	18.02	18.27	18.50	18.72	18.92
3	11.24	11.47	11.68	11.87	12.05	12.21	12.36	12.50	12.63
4	9.233	9.418	9.584	9.763	9.875	10.00	10.12	10.23	10.34
5	8.208	8.368	8.512	8.643	8.764	8.875	8.979	9.075	9.165
6	7.587	7.730	7.861	7.979	8.088	8.189	8.283	8.370	8.452
7	7.170	7.303	7.423	7.533	7.634	7.728	7.814	7.895	7.972
8	6.870	6.995	7.109	7.212	7.307	7.395	7.477	7.554	7.625
9	6.644	6.763	6.871	6.970	7.061	7.145	7.222	7.295	7.363
10	6.467	6.582	6.686	6.781	6.868	6.948	7.023	7.093	7.159
11	6.326	6.436	6.536	6.628	6.712	6.790	6.863	6.930	6.994
12	6.209	6.317	6.414	6.503	6.585	6.660	6.731	6.796	6.858
13	6.112	6.217	6.312	6.398	6.478	6.551	6.620	6.684	6.744
14	6.029	6.132	6.224	6.309	6.387	6.459	6.526	6.588	6.647
15	5.958	6.059	6.149	6.233	6.309	6.379	6.445	6.506	6.564
16	5.897	5.995	6.084	6.166	6.241	6.310	6.374	6.434	6.491
17	5.842	5.940	6.027	6.107	6.181	6.249	6.313	6.372	6.427
18	5.794	5.890	5.977	6.055	6.128	6.195	6.258	6.316	6.371
19	5.752	5.846	5.932	6.009	6.081	6.147	6.209	6.267	6.321
20	5.714	5.807	5.891	5.968	6.039	6.104	6.165	6.222	6.275
24	5.594	5.683	5.764	5.838	5.906	5.968	6.027	6.081	6.132
30	5.475	5.561	5.638	5.709	5.774	5.833	5.889	5.941	5.990
40	5.358	5.439	5.513	5.581	5.642	5.700	5.753	5.803	5.849
60	5.241	5.319	5.389	5.453	5.512	5.566	5.617	5.664	5.708
120	5.126	5.200	5.266	5.327	5.382	5.434	5.481	5.526	5.568
∞	5.012	5.081	5.144	5.201	5.253	5.301	5.346	5.388	5.427

Source: Reprinted with permission from Table B.5, Zar, J.H. (1996). *Biostatistical Analysis* (3rd ed.). Upper Saddle River, NJ: Prentice-Hall, Inc.

Table D.3d. Critical values of the *q* distribution. Alpha = 0.05.

v	p =38	40	50	60	70	80	90	100
1	68.26	68.92	71.73	73.97	75.82	77.40	78.77	79.98
2	19.11	19.28	20.05	20.66	21.16	21.59	21.96	22.29
3	12.75	12.87	13.36	13.76	14.08	14.36	14.61	14.82
4	10.44	10.53	10.93	11.24	11.51	11.73	11.92	12.09
5	9.250	9.330	9.674	9.949	10.18	10.38	10.54	10.69
6	8.529	8.601	8.913	9.163	9.370	9.548	9.702	9.839
7	8.043	8.110	8.400	8.632	8.824	8.989	9.133	9.261
8	7.693	7.756	8.029	8.248	8.430	8.586	8.722	8.843
9	7.428	7.488	7.749	7.958	8.132	8.281	8.410	8.526
10	7.220	7.279	7.529	7.730	7.897	8.041	8.166	8.276
11	7.053	7.110	7.352	7.546	7.708	7.847	7.968	8.075
12	6.916	6.970	7.205	7.394	7.552	7.687	7.804	7.909
13	6.800	6.854	7.083	7.267	7.421	7.552	7.667	7.769
14	6.702	6.754	6.979	7.159	7.309	7.438	7.550	7.650
15	6.618	6.669	6.888	7.065	7.212	7.339	7.449	7.546
16	6.544	6.594	6.810	6.984	7.128	7.252	7.360	7.457
17	6.479	6.529	6.741	6.912	7.054	7.176	7.283	7.377
18	6.422	6.471	6.680	6.848	6.989	7.109	7.213	7.307
19	6.371	6.419	6.626	6.792	6.930	7.048	7.152	7.244
20	6.325	6.373	6.576	6.740	6.877	6.994	7.097	7.187
24	6.181	6.226	6.421	6.579	6.710	6.822	6.920	7.008
30	6.037	6.080	6.267	6.417	6.543	6.650	6.744	6.827
40	5.893	5.934	6.112	6.255	6.375	6.477	6.566	6.645
60	5.750	5.789	5.958	6.093	6.206	6.303	6.387	6.462
120	5.607	5.644	5.802	5.929	6.035	6.126	6.205	6.275
∞	5.463	5.498	5.646	5.764	5.863	5.947	6.020	6.085

Source: Reprinted with permission from Table B.5, Zar, J.H. (1996). *Biostatistical Analysis* (3rd ed.). Upper Saddle River, NJ: Prentice-Hall, Inc.

APPENDIX E – STUDENT INVESTIGATIONS

Name _____

1. What is the specific **problem** to be **posed**?

2. Describe the specific **problem solving** approach you plan to use:

   The Specific Question

   The Hypotheses

   The Experimental Design

3. Record your **results** below:

4. Perform the appropriate **statistical analyses**.

5. What **conclusions** can be drawn?

6. List all **references** below:

7. Present your findings in the form of a **poster presentation**.

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